(NASA-CP-2336) NASA SPACE BIOLOGY PROGRAM: 9TH ANNUAL SYMPOSIUM Abstracts Only (National Aeronautics and Space Administration) 128 p HC A07/MF A01 CSCL 06C G3/51

N85-26054 THRU N85-26106 Unclas 17080

NASA Conference Publication 2336

NASA Space Biology Program

Annual Symposium

Thora W. Halstead, *Chairman*
NASA Office of Space Science and Applications
Washington, D.C.

Abstracts of a program review held at
Harpers Ferry, West Virginia
November 6–9, 1984

National Aeronautics
and Space Administration

Scientific and Technical
Information Branch

1985

FOREWORD

The "9th Annual Symposium of the NASA Space Biology Program" was held in Harpers Ferry, West Virginia on November 6-9, 1984. The activities included two and a half days of presentations by Space Biology principal investigators and an evening of poster session presentations by Research Associates. This symposium continues to be the key U.S. annual symposium in gravitational physiology. Over 100 scientists attended this year's meeting.

I want to thank Donald R. Beem and Louise Salmon for their help in coordinating the symposium.

Thora Halstead
February 1985

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AIBS
GRAVITATIONAL/SPACE BIOLOGY
NINTH ANNUAL SYMPOSIUM
November 6-9, 1984
Harpers Ferry, West Virginia

AGENDA

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TUESDAY, 6 November 1984

8:00-10:00 p.m. No Host Mixer - Restaurant

WEDNESDAY, 7 November 1984

8:00 a.m. OPENING REMARKS - Chairman, Robert W. Krauss

8:05 a.m. NASA Life Sciences - Arnauld E. Nicogossian
8:15 a.m. Space Biology Program - Thora W. Halstead
8:30 a.m. Life Sciences Flight Program - Tom W. Perry
8:45 a.m. Space Flight Experiment Conditions - Lynn Griffith
9:15 a.m. U.S. Space Station - Mitchell Rambler

9:30 a.m. BREAK

9:45 a.m. Society for Gravitational and Space Biology

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12:00 - 1:15 p.m. LUNCH

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7:00 - 8:30 p.m. DINNER BUFFET

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FRIDAY, NOVEMBER 9, 1984

8 a.m. to Noon — SESSION IV: SMALL DISCUSSION GROUPS

Purpose: Identification of Research Status and Future Research Requirements

Animal Gravity Receptors and Transduction
Chairperson - Muriel D. Ross

Plant Gravity Perception and Transduction
Chairperson - Robert E. Cleland

Plant Structure, Growth and Development
Chairperson - Tom K. Scott

Biological Support Structures and the Role of Calcium
Chairperson - Claude D. Arnaud

Gravity Sensitive Systems - Mechanisms and Responses
Chairperson - W. Eugene Yates

Mechanisms of Plant Responses to Gravity
Chairperson - C. Herbert Ward

Animal Development
Chairperson - Lewis D. Smith

Noon — GENERAL MEETING ADJOURNED

1:00 p.m. — AIBS Space Biology Panel (Executive Session)

6:00 p.m. — Dinner

7:00 p.m. — Continue Session

9:00 p.m. — ADJOURN

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SESSION I: GRAVITY PERCEPTION

A. ANIMAL GRAVITY RECEPTORS AND TRANSDUCTION

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Mammalian Gravity Receptors: Structure and Metabolism

Muriel D. Ross

The University of Michigan

NASA Grant NSG-9047

Goal

The long-term goal of this research is to elucidate calcium metabolism in mammalian gravity receptors. To accomplish this objective it is necessary to study both the mineral deposits of the receptors, the otoconia, and the sensory areas themselves, the saccular and utricular maculas. The main focus of this research project has been to elucidate the natures of the organic and inorganic phases of the crystalline masses, first in rat otoconia but more recently in otoliths and otoconia of a comparative series of vertebrates. Some of our recent ultrastructural findings in rat maculas, however, have prompted a more thorough study of the organization of the hair cells and innervation patterns in graviceptors. The two topics are considered separately below.

Otoconial findings

A comparative, ultra-high resolution transmission electron microscopic study of fish otolith, frog otoconia and rat otoconia was reported upon in 1983. This study showed that none of the otoconia investigated were single crystals as had been suggested by prior crystallographic studies, nor were otoliths polycrystalline. All were composites of highly ordered crystallites. In rat otoconia, which contain calcite, the crystallites were typically ~80 nm in broadest diameter and had some sharp edges. This and other findings we obtained indicated that organic material was important to the seeding and growth of the unusual crystallites included in otoconia, and was essential for ordering them into a final form mimicking that of a single crystal.

A further finding was that certain hexagonal diffraction patterns (001 faces) obtained from calcitic (rat) and aragonitic (frog) otoconial fragments sometimes were so similar that they could not be told apart. This prompted a comparative study of otoconia that included specimens from alligators (American) and turtles, which evolved from the same vertebrates that served as ancestors of birds and mammals, respectively. This study showed that the calcitic configuration occurs in the utricle of the turtle and in all the graviceptors of the alligator, although saccular otoconia of the alligator showed both calcitic and aragonitic forms. Whether or not the calcitic-appearing otoconia actually contain calcite has not yet been determined. However, the findings promted the deeper questions: Precisely what determines whether calcite or aragonite is deposited in a particular gravity receptor? What, if any, difference does this make in the functioning system? and Why is ordering the crystallites into a form mimicking that of a single crystal important?

To begin to answer these questions, we have continued our analysis of the organic phase of inner ear minerals by use of high-performance liquid chromatography (HPLC). Our findings are that rat otoconial complexes (otoconia and otoconial membranes) are high in acidic and low in basic amino acids. They also contain small, relatively insignificant amounts of proline and hydroxyproline. Through collaboration with Dr. Fulvio Perini of the Department of Pharmacology, we were able to analyze the organic material for their carbohydrate composition. We demonstrated that otoconial organic material contains relatively large amounts of glucosamine and galactosamine as well as galactose, and smaller quantities of mannose. The findings could indicate the presence of glycoproteins containing N-linked oligosaccharides, possibly with a repeating galactose-N-acetylglucosamine. In a still more recent study in which we carried

out an analysis for gamma carboxyglutamic acid, which is commonly found in biomineralized materials, our results were negative.

A previous finding in earlier work with polyacrylamide gels showed the presence of a protein of about the same molecular weight (~17,000 M.W.) as calmodulin (~16,800). We have now pursued this by submitting samples of otoconial complexes to radioimmunoassay for calmodulin. The sensitivity of the assay was 55/pg/ml, while the sensitivity of Coomassie blue staining of the gels is ~10 ng. The radioimmunoassay was, therefore, approximately 200X more sensitive. The results were negative, indicating that the presence of calmodulin in otoconial complexes is unlikely.

The results of our HPLC analysis are exciting because otoconial complexes, like other biomineralized materials (such as shells and otoliths) that contain a polymorph of calcium carbonate, have organic material that is rich in acidic amino acids. Moreover, the carbohydrate findings indicate that the organic matrix is, or contains, glycoprotein(s). In many other biomineralizing systems, it is a soluble, highly sulfated acidic glycoprotein that is considered to be important in the seeding and growth of crystallites, and in inhibiting crystallite growth beyond a specific size. It is thought that the acidic, aspartic acid residues of the glycoprotein attract calcium, then carbonate ions, to seed a crystallite by ionotropy. The crystallite grows to a certain size whereupon acidic glycoprotein in the surrounding medium is adsorbed, stopping further growth of that small crystal. Free acidic, aspartic acid residues of the adsorbed glycoprotein are, however, able to attract calcium ions to repeat the process, enabling the biomineralized material to grow in total size. While not all aspects of otoconial growth and inhibition are thus explained, the hypothesis would fit the nearly simultaneous deposition of organic and inorganic materials observed in developing otoconia.

Ultrastructural Studies of Rat Graviceptors

Our ultrastructural findings indicate that type I hair cells of both maculas are largely organized in clusters, and that type II hair cells receive their afferent innervations from calyces (the calyx leans against the type I cell) or from collaterals of calyces or their afferent nerves. Because type II hair cells are linked to more than one type I cell cluster, they may provide a means of communication between clusters.

We have also described an intramacular system of efferent-type nerve fibers and terminals that springs largely from calyces but partly from afferent nerves supplying calyceal terminals to type I hair cells. This work, based largely upon serial section reconstructions, is being pursued to determine whether another system of efferents, of central origin, exists as has been described in the literature. Regardless of the outcome of this research, the findings obtained thus far provide ultrastructural evidence for peripheral processing of sensory information in graviceptors. The intramacular system of efferent-type fibers and terminals may provide a basis for peripheral adaptation to a constant stimulus and for adaptation to a novel gravitational environment, such as microgravity.

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Effects of Weightlessness of *Aurelia* Ephyra Differentiation and Statolith Synthesis

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NASA Grant #NAGW-530

Introduction: *Aurelia* polyps are especially suited for space flight experiments because they are very small (2-4 mm), form ephyrae with gravity sensing structures in 6-7 days, and can be reared easily and inexpensively in the laboratory. During iodine-induced metamorphosis (Spangenberg, 1967), ephyrae develop in sequential order from the oral to the aboral end of the polyps. Eight sites of gravity receptors (rhopalia) form per ephyra. These structures have sacs of statoliths at their distal eand, which are composed of calcium sulfate dihydrate (Spangenberg and Beck, 1968). Only one statolith forms per cell (statocyte)and the cells collect at the distal end of the rhopalia forming statocysts. Rhopalia with statocysts are necessary for the righting reflex of swimming medusae.

Using the *Aurelia* Metamorphosis Test System (Spangenberg, 1984) for the past eight months, we have been investigating the effects of clinostat rotation in the horizontal and vertical planes on the development of ephyrae and the synthesis of their statoliths.

Methods: In order to study the effects of clinostat rotation on metamorphosing polyps, we had to build a clinostat (based on the design of a clinostat provided by Dr. C. Ward) and we had to develop a method for attaching the aquatic organisms to the clinostats. Initial testing was done to determine whether the polyps would remain attached to the tubes containing artificial sea water (ASW) in the same position during clinostat rotation for seven days. While a few polyps remained attached, most did not. We therefore tested several materials to serve as spikes for impaling the small polyps. Cactus spines were chosen for this purpose because of their rigidity, extreme thinness, and non-toxic nature. The cactus spines were embedded in the center of clear plastic autoanalyzer capsules in paraffin wax. The polyps were impaled on the spines head downwards and the spines topped with a small piece of wax. The capsules were covered with caps filled with paraffin (to reduce air bubbles in the capsules) and sealed with pressure sensitive tape. The capsules were tightly secured in a glass tube nine inches long and the tube was tightly attached to the shaft of the clinostat so that the polyp was situated on the axis of rotation of the clinostat.

Three tests were run using organisms impaled through their mouths and out their stalks. For each of the three tests, groups of six polyps (one per capsule) were treated as follows: (1) rotated in a horizontal plane at ¼ rpm; rotated in a vertical plane at ¼rpm; kept stationary in a vertical position; and kept stationary in a horizontal position. After 6-7 days at 27°C, the polyps had formed ephyrae in all of the groups and the ephyrae were removed from the capsules and placed in a wet film. The excess ASW was removed from the wet film to flatten

the animals so that the numbers of statoliths per rhopalium per ephyra could be counted and recorded. Statistical analyses were done on the data obtained using an ANOVA and the Student-Newman-Keuls test.

Results: The polyps strobilated in all of the groups tested, giving rise to numerous ephyrae. Ten ephyrae from each group were selected at random for microscopic examination. Comparison of the numbers of statoliths formed by the ephyrae which developed during rotation in the horizontal plane with controls which had either rotated in the vertical plane or were kept stationary during metamorphosis, revealed that those ephyrae from the clinostat rotated in the horizontal plane had significantly fewer statoliths than ephyrae from the conrol treatments.

Discussion: These results demonstrate that clinostat rotation at the ¼ rpm speed does not inhibit metamorphosis of polyps to form ephyrae. The finding that the number of statoliths formed is significantly lower in organisms which developed during clinostat rotation in the horizontal plane indicates that continuous disorientation of the organisms with respect to gravity may have caused fewer statocytes to initiate mineralization or may have caused fewer statocytes to differentiate. Further studies are needed to determine the specific cause of the reduced numbers of statoliths in the ephyrae which had developed during rotation in the horizontal plane.

The discovery of an effect of ¼ rpm clinostat rotation in a horizontal plane on jellyfish polyps undergoing metamorphosis is consistent with the findings of Tremor and Souza (1972) who used this speed of horizontal clinostat rotation to study developing fertilized eggs of Rana pipiens and Xenopus laevis. These authors found that ¼ rpm rotation for 5 days at 18°C produced a significantly increased number of commonly occurring abnormalities.

Little is known about factors which affect the number of statoliths made per animal during metamorphosis of Aurelia, but these results suggest that the influence of gravity may be an important factor. The exposure of metamorphosing Aurelia to the microgravity environment of outer space could be used to establish whether gravity is directly involved in the devleopment of ephyrae, their gravity receptors and their statoliths.

References:

Spangenberg, D. B. 1984. Use of the Aurelia Metamorphosis Test System to Detect Subtle Effects of Selected Hydrocarbons and Petroleum Oil. Mar. Environ. Res. 14:281-303.

Spangenberg, D. B. and Beck, C.W. 1968. Calcium Sulfate Dihydrate Statoliths in Aurelia. Trans. Amer. Microsc. Soc. 87:329-335.

Spangenberg, D. B. 1967. Iodine Induction of Metamorphosis in Aurelia. J. Exp. Zool. 165:441-450.

Tremor, J.W. and Souza, K.A. 1972. The Influence of Clinostat Rotation on the Fertilized Amphibian Egg. Space Life Sciences 3:179:191.

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Gravity Perception in a Cladoceran-Zooplankter: Anatomy of Antennal Socket Setae of Daphnia magna

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NASA Grant NAGW 643

Night orientation in Daphnia magna was recently associated, in our laboratory, with setae on the basal socket of the swimming antennae. Daphnids are suspected of maintaining nocturnal equilibrium by monitoring the gravity vector through upward setal deflections caused by sinking between antennal swimming strokes. Setae appear to be hydrodynamic rheoceptors that sense the gravity vector indirectly by mechanoreceptivity to the direction and velocity of water currents. Anatomical studies further support this hypothesis. Neuroanatomical stains have revealed i) cell bodies at the base of the setal shafts, ii) dendritic connections through to the distal ends of the shafts, and iii) axonal tracts around the antennal socket connecting with an additional cell body and continuing toward the brain. Thin sectioning of the setae and their bases has also indicated the presence of cell bodies. These anatomical observations combined with previous scanning electron microscopy studies suggest that the setae are similar to mechanorecptors and propreceptors used by higher crustaceans to sense water currents and gravity, and maintained balance. Current transmission electron microscopy investigations are being conducted to discover the ultrastructure of this sensory apparatus.

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GRAVITY RECEPTION AND CARDIAC FUNCTION IN THE SPIDER

N85-26058

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NASA Grant NAGW-242

Our research has exposed the following features of the arachnid gravity system:

1). The absolute threshold to hyper-gz is quite low. We interpret this as indicating fine proprioceptive properties of the lyriform organ, the Gz/vibration detector.

2). The neurogenic heart of the spider is a good dependent variable for assessing its behavior to Gz and other stimuli which produce mechanical effects on the exoskeleton.

3). Not only is the cardiac response useful to the investigator but it is now understood to be an integral part of the system which compensates for the consequences of gravity in the spider (an hydraulic leg extension).

4). A theoretical model has been proposed in which a mechanical amplifier, the leg lever, converts a weak force (at the tarsus) to a strong force (at the patella), capable of compressing the exoskeleton and consequently the lyriform receptor.

Throughout this work we have noted the following responses of the spider cardio-vascular system. These are:

a. changes in rate (tachycardia) and amplitude as a function of Gz
b. a bradycardia (slowing of the beat) at the immediate onset or offset of the Gz stimulus
c. comparable responses of the cardio-vascular system when vibrations are delivered to the substrate

During the latter portion of the grant period it was decide to pursue the investigation of the bradycardia. This aspect of the spider cardiac response is highly suggestive of inhibition in the CNS and specifically an inhibition evoked by sensory processes. Vibratory stimuli applied to the substrate are effective in eliciting an inhibition of the pulse. This reflex is suggestive of the "bradycardia of attention" easily demonstrated in the vertebrate animals and human beings. An inhibition of the heartbeat may function to improve the signal-to-noise ratio in stimulus detection. Thus, preparing the organism for action.

A variety of evidence points to the lyriform organ as the source of inhibitory and excitatory influences in the spider nervous system. The inhibitory nerves to the pacemaker arise within the neuropile to which the lyriforms communicate. The only environmental, i.e. stimulus, condition in which the exogenous forces affecting the Gz receptor are removed is in the micro-gravity of orbital flight. Therefore we can make a straightforward, simple test of the hypothesis that the functional removal of weight results in the reduction of inhibition of the neurogenic heart. In the weightless situation the heartrate will increase, and when a vibration is applied to the legs inhibitory control will be regained and the heartrate will decrease.

B. PLANT GRAVITY PERCEPTION AND TRANSDUCTION

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D5

AMYLOPLAST SEDIMENTATION KINETICS IN CORN ROOTS

A. Carl Leopold and Fred Sack
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NASA NSG-NAGW-3

Introduction. Knowledge of the parameters of amyloplast sedimentation is crucial for an evaluation of proposed mechanisms of root graviperception. Early estimates of the rate of root amyloplast sedimentation were as low as 1.2 μm/min (Iversen et al. 1968 Phys. Pl. 21:811) which may be too slow for many amyloplasts to reach the vicinity of the new lower wall within the presentation time. On this basis, Haberlandt's classical statolith hypothesis involving amyloplast stimulation of a "sensitive surface" near the new lower wall has been questioned (e.g. Volkmann and Sievers, 1979, Encyc. Pl. Phys.). The aim of our recent research was to determine the kinetics of amyloplast sedimentation with reference to the presentation time in living and fixed corn rootcap cells as compared with coleoptiles of the same variety.

Methods. For determination of the presentation time, seedlings were rotated 90° for various periods and then returned to a vertical orientation; curvature was recorded 20 min. after the end of gravistimulation. Determinations of amyloplast sedimentation were made using amyloplast angle with respect to the root axis; this angle was measured from photomicrographs of fixed and sectioned tissue using a Zeiss MOP2 image analyzer interfaced with an Apple computer. Living tissue sections were obtained using a Vibratome and the rootcap cells were examined with Nomarski optics on a horizontal microscope coupled to a high resolution video camera and recorder.

Results and Discussion. (1) Primary corn roots have a 4.1 min presentation time compared to 40 s for corn coleoptiles of the same variety.

(2) The determination of amyloplast location as an angle with respect to the root axis is a sensitive measure of amyloplast movement and sedimentation.

(3) The relationship between mean amyloplast angle and the logarithm of the time of gravistimulation is linear. This linearity permits extrapolation to determine the minimal time for gravity induced movement of amyloplasts to be 6 sec.

(4) The sedimentation front (i.e. lead amyloplasts close to the wall) surpasses the mean cell corner angle 19 sec. after the start of gravistimulation. The mean angle for the entire population of amyloplasts exceeds the corner angle at 2.2 min. The kinetics are similar for all amyloplasts close to the wall. Thus many amyloplasts reach the vicinity of the new lower wall well within the presentation

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time. Rapid sedimentation within the presentation time has also recently been found in corn coleoptiles (Sack et al., 1984, Planta, 161:459).

(5) Comparable kinetics were observed in living rootcap cells.

(6) These kinetics are consistent with several hypothetical explanations of the mechanism of amyloplast action including Haberlandt's statolith hypothesis.

(7) Distinct particle saltations (typically moving at 250-400 $\mu m/min^{-1}$) occur in rootcap central cells, and to our knowledge, this constitutes the first report of cytoplasmic streaming in these cells. This streaming can affect amyloplast position and sometimes cause the plastids to rise up in the cytoplasm. However, the effect of streaming on sedimentation is much more dramatic in coleoptile cells which have more vigorous streaming.

(8) Amyloplast sedimentation velocities have been measured to be up to 10 times faster in coleoptile cells, probably because of the faster streaming and because of the larger coleoptile amyloplasts. The ratio of calculated sedimentation velocities using Stokes' Law is comparable to the ratio of presentation times for the two organs. This reinforces the idea of a dependence of presentation time upon the velocity of amyloplast sedimentation.

Conclusions. Amyloplasts sediment to the vicinity of the new lower wall well within the presentation times for both corn coleoptiles and roots. Cytoplasmic streaming occurs and can affect amyloplast velocity and position in cells of both organs.

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Polyamines As Possible Modulators Of Gravity-Induced Calcium Transport In Plants

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Recent data from various laboratories indicate a probable relationship between calcium movement and some aspects of graviperception and tropistic bending responses (1-3). The movement of calcium in response to gravistimulation appears to be rapid, polar and opposite in direction to polar auxin transport. What might be the cause of such rapid Ca^{2+} movement? We believe that some recent data from studies on polyamine (PA) metabolism may furnish a clue.

Koenig et al. (4,5) have reported that a transient increase in the activity of ornithine decarboxylase (ODC) and titers of various PAs occurs within 60 seconds after hormonal stimulation of animal cells, followed by Ca^{2+} transport out of the cells. This activation may involve phosphorylation (6,7). Through the use of specific inhibitors, it was shown that the enhanced PA synthesis from ODC was essential not only for Ca^{2+} transport, but also for Ca^{2+} transport-dependent endocytosis and the movement of hexoses and amino acids across the plasmalemma.

In plants, we have shown that rapid changes in arginine decarboxylase (ADC) activity occur in response to various plant stresses (8,9). We postulate that physical stresses associated with gravisensor displacement and reorientation of a plant in the gravitational field could similarly activate ADC and that resultant increases in PA levels might initiate transient perturbations in Ca^{2+} homeostasis. The fact that amyloplasts, the putative graviperceptors in many plant tissues, are reported to be high in Ca^{2+} (10) suggests one possible source of Ca^{2+} which might be mobilized. This hypothesis is immediately amenable to experimental testing.

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CALCIUM AND CALMODULIN LOCALIZATION IN GRAVITROPICALLY-RESPONDING PLANT ORGANS

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NASA Grant NSG 7480

In earlier studies, we documented the rapid onset of calcium distribution asymmetry in gravitropically stimulated oat coleoptiles, using pyroantimonate to immobilize and "stain" calcium in the tissue. Here we report the results of our study of calcium distribution in gravitropically stimulated corn roots, again using antimonate to rapidly fix the calcium in the tissue. Our earlier studies also implicated calmodulin as being an important regulator of one or more of the transduction events in the stimulus-response sequence leading to gravitropism. To help clarify the potential role of calmodulin in tropistic growth, we have used radioimmunoassays to quantitate calmodulin in intact tissue and in isolated subcellular fractions and immunocytochemical methods to localize calmodulin in roots and coleoptiles. Here we report preliminary findings from these studies.

Our investigation of whether there is a redistribution of calcium in corn roots when they are induced to grow downward utilized essentially the same antimonate staining procedures we had used earlier to detect calcium redistribution changes in oat coleoptiles. We found that in the region of the developing bend there was a change in the staining pattern from symmetrical staining principally localized within cells of the stele to asymmetric staining within the vacuoles in the cortical cells along the upper surface of the root. There was very little staining apparent in the walls. Although this pattern is quite different from that seen in gravitropically responding coleoptiles, it does show that an asymmetric redistribution of Ca is induced by a gravitropic stimulus in roots as it is in shoots. Since this response occurs within 10 min, at least 5 min before any visible bending, it could play a role in the regulation of root gravitropism.

We used two different general approaches to localize calmodulin in plant tissue: radioimmunoassay of its content in tissue and in purified subcellular organelles, and immunocytochemical detection of it in roots and coleoptiles. Our radioimmunoassay results indicated that calmodulin is present in large quantities in plant cells and that it is specifically associated with mitochondria, etioplasts and nuclei. In collaboration with M. Terry of L.S.U., we also assayed an extract of soluble wall proteins and found that over 1% of these proteins was calmodulin. Controls indicated that this calmodulin was not cytoplasmic in origin.

The initial immunocytochemical localizaton studies were carried out by Daye Sun from our laboratory in collaboration with Dr. C. Lin at Baylor Medical School in Houston. They used rabbit antiserum prepared against bovine brain calmodulin together with a peroxidase-labeled second antibody to detect calmodulin in corn roots. Reaction product from

anti-calmodulin was found mainly in the root cap cells, moderately in metazylem elements, in some cells in the stele surrounding metaxylem elements and in cortical cells.

These studies are being continued by M. Dauwalder in our laboratory, using improved techniques to test and extend the initial findings. Corn root tips and coleoptiles are being examined at both the tissue and cellular levels using a Lowicryl K4M embedding medium. This polar medium is suitable for both the relatively thick sections (5 um) that are needed for tissue staining with concommitant resolution by light microscopy at the cellular level. Thin sections of the same samples can then be used for electron microscopic localization. After the sections are treated with anticalmodulin, the antibody is localized at the cellular and tissue level by fluorescence visualization with Protein A-FITC. Adjacent thin sections are stained with Protein A-gold and the ultracellular pattern is determined. Our initial findings tend to corroborate those of Sun and Lin, except that we do not observe as high a concentration of anti-calmodulin stain in the root cap. We hope this approach will help to resolve whether calmodulin is localized in the wall as well as in intracellular spaces.

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THE INTERACTION OF CALCIUM AND AUXIN IN THE GRAVITROPIC RESPONSE OF ROOTS

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Recent research has implicated calcium as a mediator of root gravitropism. Evidence in support of this possibility includes the findings that: 1. Treatment of root caps with calcium chelating agents such as EDTA results in a loss of gravitropic sensitivity (1). 2. Gravitropic sensitivity in EDTA-treated roots is restored upon withdrawal of EDTA and treatment with calcium (1). 3. Artificial establishment of a calcium gradient across the caps of intact roots or across the tips of decapped roots induces gravitropic-like curvature toward the high side of the calcium gradient (1). 4. Calcium is rapidly transported across root caps, and gravistimulation causes this transport to become strongly polarized in the downward direction (2). 5. Chemical inhibitors of gravitropism such as naphthylphthalamic acid or triiodobenzoic acid inhibit gravi-induced polar calcium movement (3).

Although these findings indicate that calcium may play an important role in the gravitropic response of roots, they focus primarily on the sensory region of graviresponsive roots (the cap). The results leave open the question of the role that calcium redistribution may play in the responding region of the root (the elongation zone), and they do not shed light on the potential connection between calcium and auxin redistribution in the elongation zone. According to the classical Cholodny-Went theory, root gravitropism results from a stimulus-induced accumulation of auxin to growth-inhibitory levels in cells on the lower side of the elongation zone. With this theory in mind we have examined: 1. the effect of gravity on calcium movement across the elongation zone, 2. the effect of gravity on auxin movement across the elongation zone, and 3. the effect of calcium on auxin movement across the elongation zone.

Calcium movement across the elongation zone was tested by applying ^{45}Ca to one side (ca. 4 mm from tip) of the roots of 3-day-old light-grown seedlings of the maize cultivar B73 X Missouri 17. After 45-90 min, radioactivity was determined in the tissue halves adjacent to and opposite from the donor. Using this method, no polarity of ^{45}Ca movement was observed across the elongation zone of vertical roots. In contrast, a polarity of about 2-fold was observed for ^{45}Ca movement across gravistimulated roots, with preferential movement toward the lower side. Gravi-induced polar ^{45}Ca movement across the elongation zone was not found in non-responsive roots of dark-grown seedlings or in roots from which the caps had been removed.

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The movement of auxin across the elongation zone of intact roots was measured by applying ^{3}H-IAA to one side of the elongation zone and measuring accumulation of label in an agar receiver placed directly across from the donor on the opposite side of the elongation zone. The influence of calcium on the movement of ^{3}H-IAA was determined by incorporating calcium chloride (10 mM) into the receivers. In vertically oriented roots movement of labeled ^{3}H-IAA across the elongation zone was slight and was not enhanced by incorporating calcium chloride into the receiver block. In horizontally oriented roots, movement of label across the root was readily detectable, and movement to a receiver on the bottom was about 3-fold greater than movement in the opposite direction (Fig. 1). This polarity was abolished in roots from which the caps were removed prior to gravistimulation.

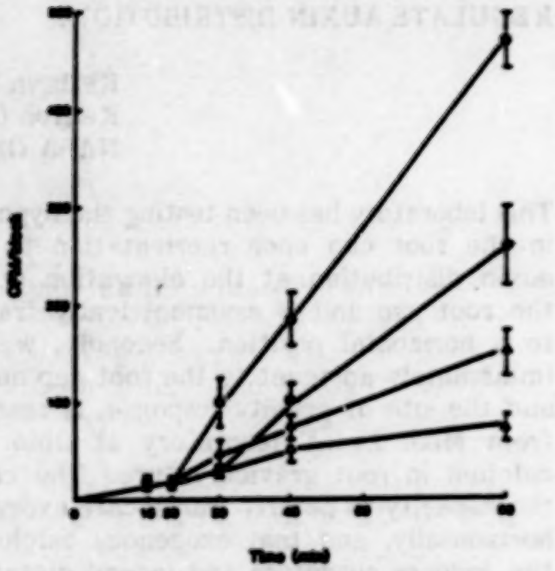

Fig. 1. Time course of transport of label from tritiated IAA across the elongation zone of horizontally oriented maize roots. Transport from bottom to top, no calcium added to receiver (▲-▲). Transport from top to bottom, no calcium added to receiver (○-○). Transport from bottom to top, 10 mM calcium chloride added to receiver (▲-▲). Transport from top to bottom, 10 mM calcium chloride added to receiver (○-○). Each point represents the average of five experiments ± standard deviation.

When calcium chloride was incorporated into the receivers, movement of label from ^{3}H-IAA across horizontally-oriented intact roots was increased about 3-fold in both the downward and upward direction (Fig. 1). The ability of calcium to enhance the movement of label from ^{3}H-IAA increased with increasing calcium concentration in the receiver up to 5-10 mM calcium chloride. With the inclusion of calcium chloride in the receiver blocks, gravity-induced polar movement of label into receiver blocks from applied ^{3}H-IAA was detectable within 30 min, and asymmetric distribution of label within the tissue was detectable within 20 min. The results indicate that gravistimulation induces a physiological asymmetry in the auxin transport system of maize roots and that calcium increases the total transport of auxin across the root. Gravistimulation is apparently necessary for the enhancing effect of calcium on lateral auxin movement, and it is possible that the preferential downward movement of calcium across the elongation zone of gravistimulated roots plays a role in establishing the auxin asymmetry proposed to cause positive gravitropic curvature.

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TRANSDUCTION OF THE ROOT GRAVITROPIC STIMULUS: CAN APICAL CALCIUM REGULATE AUXIN DISTRIBUTION?

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This laboratory has been testing the hypothesis that calcium, asymmetrically distributed in the root cap upon reorientation to gravity, affects auxin transport and thereby auxin distribution at the elongation zone. We are assuming that calcium exists in the root cap and is asymmetrically transported in root caps altered from a vertical to a horizontal position. Secondly, we are assuming that the meristem, the tissue immediately adjacent to the root cap and lying between the site of gravity perception and the site of gravity response, is essential for mediation of gravitropism. Evidence from Mike Evans' laboratory at Ohio State University has strongly implicated tip calcium in root gravicurvature. The capstone evidence being that the root cap has the capacity to polarly translocate exogenous calcium downward when tissue is oriented horizontally, and that exogenous calcium, when supplied asymmetrically at the root tip, induces curvature and indeed dictates the direction of curvature in both vertical and horizontal corn roots (Lee, et al, 1983a,b).

We are carefully investigating the possible effect of calcium on basipetal transport of 3H-indoleactic acid (3H-IAA) from the meristem to the elongation zone and its lateral distribution. Basipetal translocation of IAA is likely to be the source of delivery of auxin to the elongation zone because not only is basipetal transport well established to occur in 1 cm root tips, but this transport is faster than that moving towards the apex (Konings, 1967; Davies and Mitchell, 1972; Ohwaki and Tsurumi, 1976). Secondly, Lew Feldman (1981) has elegantly shown that the meristem has regulatory power for auxin transport in roots. Along with the location of the meristem between the root cap and elongation zone, the mediation by the meristem of auxin delivery to the elongation zone and consequently of gravitropic curvature becomes an attractive possibility.

Two-day-old corn (cv. Silver Queen) roots were decapped for these experiments to remove the assumed endogenous calcium source as well as to provide an apical surface from which auxin could be transported (because IAA is retained in and not readily translocated from the root cap). 6mm apical segments, which include the proximal portion of the elongation zone and the tissue through which transduction must occur, were used in order to maximize recovery of transported IAA in the elongation zone in the relatively short time period of 90 min. Root segments were oriented horizontally. 3H-IAA (10-25 nM) was applied to the proximal meristem in agar buffered at pH 5.2. Calcium (5-10mM) was positioned in a buffered agar block to either the upper or lower surface of the meristem and buffered receivers were placed on the upper and lower surfaces of the elongation zone, 5-6mm from the apex (Fig. 1). Results from these experiments are predicted by the hypothesis. More auxin accumulates in the elongation zone on the side corresponding to the calcium applied at the apex. The distribution of label across the elongation zone was found to be greater on the lower than upper side in controls without apical calcium while a 1:3 ratio was produced when calcium was applied to the lower apical surface (Fig 2). These results also substantiate those of Konings (1967, 1968) for intact, horizontal pea roots. Konings found basipetally translocated 14C-IAA was asymmetrically distributed in upper and lower halves of two-day-old horizontal pea roots 90 min after auxin application. The asymmetry was downward with a 1:2 ratio, was only observed when the root cap or a portion of it was present, and was strongest at the apex, diminishing in the basipetal direction. Konings concludes, in line with our hypothesis, that the root cap is essential for asymmetric distribution of auxin in root tissue and that the asymmetry arises first at the apex and proceeds towards the base.

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An alternative explanation for the asymmetry we observe in Fig. 2 is that the asymmetrical geometry of the agar blocks applied to the root would inherently produce the asymmetric radiolabel distribution. This is apparent from the controls with upper or lower buffered agar blocks replacing the calcium (data not shown; controls in Fig. 2 are without lateral blocks at the apex) which show a similar radiolabel asymmetry. However, the control asymmetry is not large enough to account for the differences observed with calcium. To examine this alternative explanation further an experiment with symmetrical blocks was performed where the apical calcium block was juxtaposed with a buffered block without calcium (Fig. 3). Here we again found radiolabel to accumulate in the lower receivers and calcium at the tip enhanced this asymmetry. However, calcium on the upper apical surface did not reverse this downward distribution (Fig. 4).

One explanation for the results from the Fig. 3 experiments is that the symmetrical geometry of agar blocks across the tip might facilitate calcium diffusion, diminishing the calcium gradient operating in Fig. 1. To examine this, methylene blue was included in the calcium block to visualize diffusion movement. None was visible. This may be due to the reduced hydrogen ion concentration at the root apex and along the lower surface consequently oxidizing the dye to its colorless state.

Our results neither prove nor disprove the hypothesis. Various alternative geometries are now being investigated to ascertain the meaning of these experiments. Increasing the resolution of the asymmetry is another objective.

Figure 1

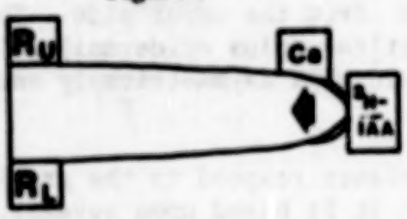


Figure 2

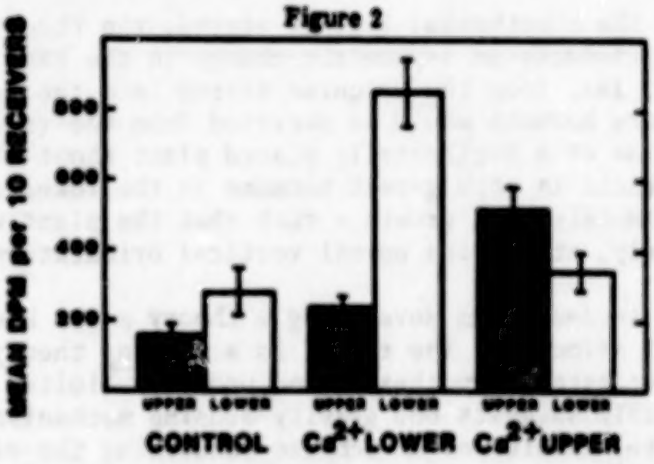


Figure 3

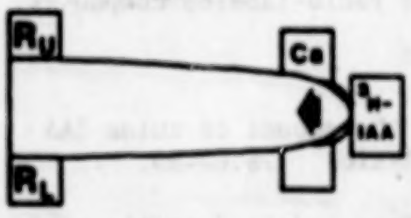
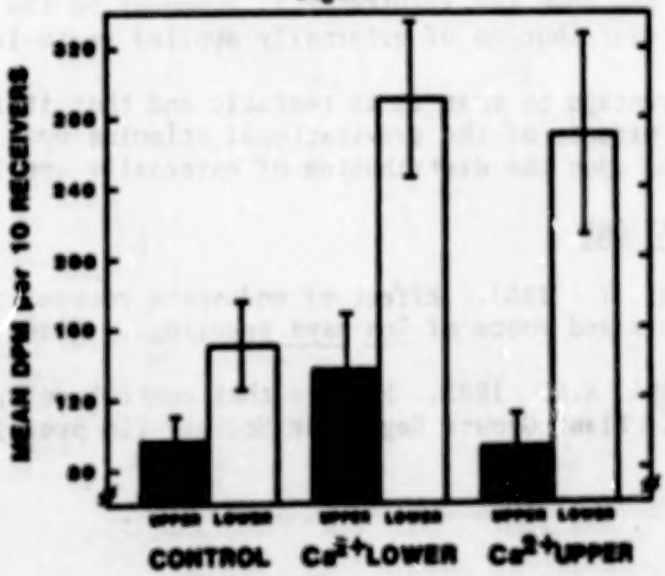


Figure 4



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ATTEMPTS TO LOCALIZE AND IDENTIFY THE GRAVITY-SENSING DEVICE OF PLANT SEEDLINGS

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Growth Hormone Asymmetry:

We believe that we have started to understand how and where the gravitational stimulus can induce a plant growth hormone asymmetry. The ingredients to this understanding are composed of several parts. The first, and most important part is the discovery that the growth hormone asymmetry develops within three minutes following the initiation of the gravitational asymmetry. The second finding is that radio-labeled compounds being transported from the seed to the shoot also show asymmetric distribution. These findings indicate to us that the target of the gravity stimulus resides primarily in the permability of the vascular tissue that regulates the supply of hormone to the surrounding tissues.

This is the hypothesis: Briefly stated, the theory is that the gravitational stimulus induces an asymmetric change in the rate of secretion of the growth hormone, IAA, from the vascular tissue into the surrounding cortical cells. Thus, more hormone would be secreted from the vascular stele proximal to the lower side of a horizontally placed plant shoot than from the upper side. This would result in more growth hormone in the lower cortical (plus epidermal) cells, and ultimately more growth - such that the plant would grow asymmetrically and, ultimately, attain its normal vertical orientation.

We have succeeded in developing a theory as to how plants respond to the gravitational stimulus. The theory is a working theory. It is based upon several, as yet untested, hypotheses, and upon our limited knowledge of one kind of plant; and it only suggests one gravity sensing mechanism. The theory is based upon the analytical results of A. Schulze concerning the effects of gravity on the distribution of the plant growth hormone, IAA, in both its free and conjugated forms, and upon the results of Y. Momonoki on the effect of the growth stimulis on the distribution of externally applied radio-labeled compounds.

Its advantage is that it is testable and that it is built upon solid knowledge of the effects of the gravitational stimulus upon the endogenous growth hormone, IAA, and upon the distribution of externally applied radio-labeled compounds.

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Role of Ca^{++} in Shoot Gravitropism

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NASA Grant NA6W-230

Recently several laboratories have reported that a Ca^{++} asymmetry develops across horizontally oriented roots and shoots. Today I will report on experiments designed to evaluate the role of Ca^{++} in straight growth and gravitropism.

A cornerstone in the argument that Ca^{++} levels may regulate growth is the finding that EGTA promotes straight growth. The usual explanation for these results is that Ca^{++} chelation from cell walls results in wall loosening and thus accelerated straight growth. This reasoning is certainly logical at first blush. Exogenous Ca^{++} does strongly inhibit growth and wall loosening and EGTA does indeed chelate and thus reduce free cell wall Ca^{++} levels. Nevertheless, an alternative explanation for EGTA-induced wall loosening exists. When EGTA chelates Ca^{++}, nitrogen bound protons are liberated. Given this fact, I believe it is reasonable to speculate that EGTA application results in wall acidification and thus acid growth. Further, I suggest Ca^{++} chelation in the absence of such acidification would be inconsequential. What is my evidence?

Recently, I examined the ability of frozen-thawed Avena coleoptile tissue (subjected to 15g tension) to extend in response to EGTA and Quin II. EGTA when applied in weakly buffered (i.e. 0.1mM) neutral solutions initiates rapid extension. When the buffer strength is increased (i.e. to 20 mM) similar concentrations of EGTA produce no growth response. This implies when EGTA liberated protons are released upon Ca^{++} chelation they can either initiate acid growth (low buffer conditions) or if "consumed" (high buffer conditions) have no effect. Thus Ca^{++} chelation in itself apparently does not result in straight growth.

To circumvent obvious criticism of these results and conclusions (e.g. the buffer strength in itself prevents wall loosening, the higher buffer strength prevents EGTA from chelating wall Ca^{++}, etc.) I employed another chelator, QUIN II. At neutral pH's QUIN II does not liberate protons upon Ca^{++} chelation. Preliminary data indicate that at both high and low buffer strengths QUIN does not stimulate straight growth. As QUIN II is reported to have an affinity for Ca^{++} similar to EGTA, it seems likely that chelation did occur but that lack of acidification circumvented a growth response. In support of this notion I have found that pretreatment with QUIN II prevented a subsequent growth response to EGTA. Addition of exogenous Ca^{++} restores the ability of EGTA to promote growth.

While Ca^{++} redistribution may not directly influence asymmetric growth (see above) some evidence exists that Ca^{++} gradients may influence auxin transport and thus indirectly play

a role in gravitropism. Often cited in support of this notion are the results of Dela Fuente and Leopold. These investigators reported that polar auxin transport is dependent on Ca^{++} and suggested that IAA and Ca^{++} transport are coupled although opposite in direction. I will present data which shows that both polar and lateral auxin transport are influenced by Ca^{++} gradients. However, it seems unlikely that there is a direct coupling between Ca^{++} and auxin movement in shoot systems.

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GRAVISTIMULUS PRODUCTION IN ROOTS OF CORN

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In many cultivars of corn if seeds are germinated and maintained in darkness the primary root fails to respond to gravity. Illumination of the root cap with white light causes the root to bend downwards.

The objectives of our work have been to determine the processes by which light initiates gravitropic bending. Using this system we hope to elucidate biochemical steps involved in the transduction of the gravity stimulus into a growth response.

Our efforts during the past year have focused on determining a possible photoreceptor in the cap for the light. For all experiments 40-44 hours old roots of corn were used. For some experiments caps from roots maintained in the dark (dark-grown) were excised and the pigments extracted. In other experiments roots were illuminated briefly with white light, returned to the dark for varying intervals of time after which the caps were excised and extracted for pigments as before. In addition to root cap tissue, we also collected the terminal mm of tissue proximal (basal) to the root cap. Terminal mm tissue was collected from both dark-grown and light-treated roots. Cap tissues (1000-1500 caps) from the various treatments were extracted in methanol, filtered and scanned in a spectrophotometer. In caps from tissues maintained in the dark we obtained spectra characteristic of carotenoids, with 3 prominent peaks in the 400-500 nm range. Similar spectra were obtained from illuminated cap tissue. From such spectra we calculated the levels of total carotenoids in cap tissues. Light causes a 50-60% increase in the levels of total carotenoids compared to levels recorded for caps maintained in continuous darkness. The maximum increase in light-stimulated carotenoids is observed in tissue provided light and then returned to the dark for 30-60 min. In tissues which were illuminated and returned to the dark for 3 hours or more, total carotenoids returned to levels observed in caps maintained in complete darkness.

Not all carotenoids follow this pattern. When the carotenoids from the cap are fractionated, we have shown that for some carotenoids light causes a reduction in total levels. One particular carotenoid which follows this pattern and which we have definitively identified with mass spectroscopy, is violaxanthin. Violaxanthin is very labile to acid, air and light, and when illuminated, rapidly (within 10 minutes) drops to a level 50-70% less than that observed in caps maintained in complete darkness. If roots are illuminated and returned to the dark for periods in excess of 3 hours, violaxanthin levels increase to that observed in dark, control root caps. On a per gram basis violaxanthin is 7 times more concentrated in the root cap compared to the adjacent basal meristem tissue (1.51 µgm vs 0.22 µg/gm fresh weight). We consider this documentation of the presence of violaxanthin within the root cap and its changes in levels following light treatment of potential significance in understanding the mechanism by which light modulates root gravitropism.

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Because of the similarities in structure of known growth regulators, specifically abscisic acid and xanthoxin, with portions of the violaxanthin molecule, it has been suggested that these growth substances normally arise from the breakdown or turnover of this carotenoid. We have shown that the light-induced disappearance of violaxanthin occurs in a time frame coincident with an increase in the levels in cap tissue of substances with growth inhibitor properties. From this work we wish to propose that one of the ways by which light may regulate root development, including aspects of gravitropism, is through the production of inhibitory growth substances arising from the turnover of carotenoids.

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A POSSIBLE EXPLANATION OF WHY GRAVITROPIC GROWTH IS DETECTED EARLIER THAN STRAIGHT GROWTH

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My grant for the past year has been for the purpose of testing an hypothesis for the mechanism of gravitropic gravity reception by seedling shoots. It was not anticipated that the project would be completed within the year, and a number of new techniques must be worked out before we can expect decisive results.

During the year I have also given thought to the processes by which gravitropism is mediated. I have been particularly interested in data from the labs of Stan Roux, Bob Bandurski and Peter Kaufman as well as some colleagues outside our Space Biology Group which established that not only component reactions but also the ultimate curvature, presumably a result of differential growth, can be observed within considerably less than 10 min after an optimally sensitive seedling is placed on its side. This is not to say that such rapid response is universally observed. However, the fact that it is sometimes observed is important because we embrace the dogmas that

1. Gravitropism is mediated by lateral migration of the auxin IAA.
2. There is a 10-min lag for induction of growth by IAA. (This lag is important because it figures heavily in attempts to explain auxin action.)

Because the intent of this workshop is to integrate and explain our collective data and discover fruitful new paths of hypothesis and experimentation, I propose to discuss how I think gravitropic curvature can be controlled by IAA and yet show a briefer lag than IAA-induced straight growth. In the process, I am going to suggest that the processes underlying growth are actually underway equally early during straight and asymmetric growth, and that the always-observed minimum straight-growth lag of 10 min is a consequence of feedbacks which act to restrain expression of growth when IAA is provided symmetrically and to accelerate expression of growth when IAA is provided asymmetrically.

What are the evidences for the rapidity of gravitropism?

1. Kaufman, Bandurski and collaborators (Plant Physiol. 74:284, 1984) and also Hild and Hertel (Planta 108:245, 1972) have reported that gravitropic curvature of coleoptiles and mesocotyls can begin within 3 - 5 min of displacement from the vertical position.
2. Bandurski and Schulze (Plant Physiol. 75:S178, 1984) have reported IAA asymmetry for gravitropically stimulated mesocotyls within 3 min, and Hertel et al. (Planta 94:333, 1970; Planta 108:245, 1972) have reported it for coleoptiles within 5 - 10 min.
3. Slocum and Roux (Planta 157:481, 1983) have reported apoplastic calcium asymmetry in gravitropically stimulated coleoptiles within 10 min; Dauwalder and Roux see it in 2 min (personal communication).
4. Tanada and Vinten-Johansen (Plant Cell Environ. 3:127, 1980) have reported that the lower sides of gravitropically stimulated hypocotyls become relatively electronegative in about 2 min.

How then can the discrepancy between lags for straight and asymmetric growth be explained? Imagine that lateral IAA transport begins immediately when a shoot is displaced to the horizontal position. Within a very short time, the IAA concentration in the upper epidermis will be lowered, while that in the lower epidermis will be raised. The epidermis is, of course, the tissue limiting seedling growth.

Imagine that the rate of proton extrusion responds immediately to the changing levels of IAA. Dramatic changes in apoplastic acidity might lag because a) the apoplast (which includes the wall) is buffered, and b) measurement of acid is usually logarithmic, minimizing the detection of subtle change. However, while it takes a lot of protons to effect a noticeable concentration change, it takes relatively few unneutralized protons to effect a substantial electrical change. Voltage differences across the tissue could thus be established rapidly (upper side negative with respect to lower side).

Imagine that Ca^{2+} within the apoplast migrates down the voltage gradient. Imagine that its migration is also impelled by development of a chemical gradient, for as protons accumulate on the lower side they displace bound Ca, and as they decrease on the upper side Ca^{2+} is bound more effectively. (This process may be further complicated by dumping of vacuolar Ca^{2+} into the apoplast - dumping kinetics have not been assessed. See Slocum and Roux, ibid.).

Inhibition of growth by apoplastic Ca^{2+} is very fast - an outside limit of 2 or 3 min has been reported by Cleland and Rayle (Plant Physiol. 60:709, 1977) and Evans (Thesis, Univ. of Calif. at Santa Cruz, 1967). Stimulation of growth by apoplastic protons is thought to be essentially without lag. The rapidly developed opposing asymmetries of H^+ and Ca^{2+} would reinforce to initiate gravitropic growth.

On the other hand, protons released symmetrically across a uniformly elongating tissue would not give rise to an electrochemical gradient for Ca^{2+} and the symmetric release of Ca^{2+} from certain presumably inert binding sites in the wall might free it to bind inhibitorily to active sites in the wall. Thus, the growth-promoting tendency of early-appearing apoplastic H^+ would be bucked and could even be cancelled by the inhibiting tendency of freed Ca^{2+}. Such a stalemate would be broken only when the level of apoplastic H^+ rose above a threshold which would depend on the abundances and binding constants of the presumably several types of Ca^{2+}-binding sites in the walls (as well as on the kinetics of vacuolar dumping).

In test of the hypothesis, measurements should be made in a single test system of the rapidity with which a) IAA asymmetry develops during gravitropic stimulation, b) asymmetric IAA causes upward curvature and gradients of voltage and Ca^{2+}, and c) asymmetric H^+ causes upward curvature and gradients of Ca^{2+}; these should be compared with measurements on straight growth induced by IAA. Then, IAA should be applied omnilaterally to a short (about 1 mm) length of the stem; by analogy with the case of lateral asymmetry, this axial nonuniformity should produce axial H^+, voltage and Ca^{2+} gradients which lead to elongation of the zone of application after about 3 min. Further, radially symmetric inhibition might be observed at the apical edge of the application zone and - if polar IAA transport were to be inhibited with naphthylphthalamic acid - at the basal edge of the zone as well. Elongation of the same morphological region should lag 10 or more min if IAA is applied to the whole length of the stem.

SESSION II. ROLE OF GRAVITY IN DEVELOPMENT

A. PLANT GROWTH AND DEVELOPMENT

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DAYLILY AS A SYSTEM TO STUDY EFFECTS OF SPACE FLIGHT ON PLANT DEVELOPMENT

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NASA Grant NSG-7270

In past studies carried out in this laboratory, Hemerocallis cv. 'Autumn Blaze' suspension-derived protoplasts were reproducibly isolated, collected, induced to regenerate their walls and to divide and form small granular cell clusters. The clusters when placed in appropriate media and taken through a sequence of media changes multiplied, organized and formed plantlets. Plantlets were generated from protoplasts by methods developed by us originally for the regeneration of carrot and daylily plantlets from suspension cultures. The methods worked out and described earlier are now reasonably convenient to carry out and give reproducible results for material at given stages of development. Even so, we are constantly seeking to refine and improve on details. When cultures are used in certain stages of development, competent cells generate a very large number of plantlets. The production of daylily plants from protoplast cultures demonstrates that the protoplasts retain their totipotent character.

But the totipotent character is not retained indefinitely and through all stages of development. From the perspective of our totipotent culture system, we start off with cells which show no obvious or diagnostic signs of differentiation. They are then switched to another medium. This medium initiates the developmental pathway which, in turn, leads to the beginning of organized growth. An additional medium change permits the material to assume fully developed organization. Prior to our work, there were no reports of successful isolation and culture of totipotent protoplasts from daylily or any other perennial monocotyledonous plant. In addition, insufficient attention had been paid to the description and documentation of the occurrences in the very earliest stages of protoplast culture. In general, the view has generally been that the steps involved in culturing protoplasts from any source material involves: 1) isolation of protoplasts which remain as single entities; 2) the regeneration of a new cell wall around the protoplasts; 3) first cell division; 4) further cell divisions. In this way a cluster of cells originating from a single protoplast, a "protoclone" in a manner of speaking, would arise. This cell cluster would then be induced by any number of sequences to form a plant identical to the original plant from which the protoplasts were derived.

Our findings show that the daylily and carrot systems contrast with the above scheme. We feel, however, that these systems with which we have been working are not unusual. Other systems in this laboratory follow this route as well. The events we are encountering may well represent more carefully notated and studied systems.

Our observations expand upon and enhance understanding of the hitherto accepted view in the regeneration process as follows:

1) Protoplasts derived from totipotent cells grown in suspension culture do not remain as single entities. Instead, they group together into aggregated protoplast masses within 24 hours after they are isolated, whereas, incompetent protoplasts derived from petal, root and leaf do not aggregate to any degree. Neither do they divide in culture;

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2) The majority of the protoplasts are in an aggregated state, and it appears that cell wall regeneration occurs more or less simultaneously in a number of protoplasts rather than in any single protoplast alone;

3) Protoplasts do not respond uniformly to culture conditions. Some of the protoplasts divide in the "textbook fashion", most do not. Budding, usually attributed to be the result of improper culture conditions, i.e. inappropriate media composition, is observed in cultures along side normal cell division. Clearly, such differences observed within a single culture vessel are more likely a result of differences existing within a protoplast population.

4) Cell clusters arising in protoplast cultures are not necessarily derived from a single protoplast and therefore strict protoclones are not readily formed.

5) Plantlets derived from protoplast cultures may be, but are not necessarily, identical to the original plant from which the culture is derived, nor are they necessarily identical in karyotype or phenotype to each other. Rigorous controls are needed to guarantee stability and we have established the parameters for doing so.

Whereas the initial intent of our protoplast experimentation was to develop a system which would permit us to work with wall-less counterparts of totipotent free cells as a sort of model for a fertilized egg cell, it is now clear that the daylily system is becoming an increasingly valuable tool with which to study any number of basic phases of higher plant development. The truly satisfying aspect of the system is that it can now be studied from a number of perspectives. In short, a system amenable to rigorous experimentation has been developed and can be used as a point of departure for studying problems of development in the space environment. This, in turn, will be a prelude to studying the effect of hypogravity on higher plant development.

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THE ROLE OF GRAVITY ON THE REPRODUCTION OF ARABIDOPSIS PLANTS

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The hypothesis tested was whether the presence of gravity is a necessary environmental factor for higher plants to complete their life cycle. In other words, can plants grow and reproduce in a micro-g environment? To test this hypothesis, Arabidopsis thaliana (L.) Heynh. "Columbia strain" plants were grown continuously for three generations in a simulated micro-g environment as induced by horizontal clinostats. Growth, development and reproduction were followed.

The rationale of selecting and growing Arabidopsis plants for three generations on clinostats were 1) a short life cycle of around 35 days, 2) the cells of third generation plants would in theory be free of gravity imprint and 3) a third generation plant would therefore more than likely grow and respond like a plant growing in a micro-g environment.

Cultural procedures were developed and defined for growing plants axenically. These procedures also had the requirements of minimum manipulation between the time of seed planting and seed harvest of the following generation. The exchange of atmospheric gases between culture atmosphere and ambient was found to be necessary if plants were to complete their life cycle. In closed cultures similar to those used in space and simulated space experiments, CO_2 accumulated to high levels (5% to 14%) and Arabidopsis did not set seeds when cultured in these gas tight closed cultures. The recent success of growing Arabidopsis from seed to seed in space may have been enhanced by the first time utilization of a "ventilated" plant chamber. Such a chamber would reduce the possible accumulation of various atmospheric and plant metabolic gases which could hinder the production of seeds. In our work to be reported, the culture tubes used permitted ample gas exchange with ambient.

Three consecutive generations of Arabidopsis were grown on clinostats. Only during harvest and planting were the seeds or plants of Arabidopsis removed from the clinostats. The time seeds or plants were off the clinostats was kept to a minimum. The overall experimental protocol was as follows. A single seed was selected to generate an ample seed stock from which three lines of plants arose. The first two lines were the controls, i.e., upright stationary, 1.0 g and vertically rotated, 1.0 g. The third line was the clinostatted or simulated micro-g treatment. Cultural conditions were 350 ft.c. of continuous light from cool white fluorescent lamps, 23 $\pm$1°C, 50-55 percent relative humidity and a horizontal clinostat rotation of 0.5 rpm. A complete nutrient media supplemented with 2.0 percent sucrose and 0.002 percent glutamic acid was used.

The results were as follows. Flower formation, seed production and seed maturation were delayed in horizontally clinostatted plants. Weight of total seeds and pods produced were also less. However, germination percentage of seeds was not significantly different from that of controls. In terms of growth, a greater production of dry mass of both roots and shoots occurred in clinostatted plants than in controls. Also more roots as compared to shoots, in dry weight, was produced by

clinostatted plants than by controls, i.e., plant produced more roots when clinostatted. Morphologically, a significantly greater number of stems developed in clinostatted plants than in controls.

These changes appear in the first generation of plants that were clinostatted. These significant differences remained relatively the same over the three generations. A decrease in vigor appeared to be present in clinostatted and the control plants as the study progressed. Cultural methods developed for these studies were primarily designed to be used for space experiments and also to be used in experiments to provide baseline data for flight experiments. Restrictions such as small volume containers, low intensity light and minimum maintenance during the experiment may have been the reasons for decline in the vigor of plants in all treatments.

The changes brought about by the clinostat treatment appear not to be artifacts but appear to be real and thus may give insight as to the plant responses during long duration micro-g exposure. For plants that were clinostatted, what might be the basis for the delay in flower development and the subsequent delay of seed formation and development? Could the delay be a result of a redistribution of nutrients and photosynthates? The redistribution concept is supported by the increase in root dry weight relative to the shoot dry weight. Or could the delay be a result of a redistribution of hormones? A notable shift of a single stem in the control plant to that of multiple stems in the clinostatted plants would add credence to a redistribution of hormones such as indole-acetic-acid and the subsequent loss of apical dominance in the original single stem. Or could there have been a delay in floral induction by the clinostat treatment such as that reported for cocklebur plants? A delay of the transition time from vegetative to reproductive growth is supported by the significant increase of root and shoot weight of clinostatted plants over those of controls. Thus some evidence is at hand indicating that gravity has a role in the reproduction of Arabidopsis plants.

To test whether changes induced by clinostatting were genetically or environmentally induced, 4th generation seeds of the Arabidopsis line in which plants were grown only on clinostats were split into two sets. One set of seeds was tested under 1.0 g conditions and the other set tested again on clinostats. Both sets of seeds were then compared to the 4th generation seeds from the line which were grown only under 1.0 g. The clinostatted plants were again significantly slower in reproduction and also had more stems. However, no significant difference could be detected between the plants of the clinostatted line grown under 1.0 g and the plant of the upright stationary line. Thus the significant difference observed in the third generation between clinostatted and non-clinostatted plants appear strictly to be due to the simulated micro-g environment and not as a result of genetic changes induced by selective pressures of the simulated micro-g environment imposed for three generation.

For the hypothesis tested, gravity was found not to be a required environmental factor for higher plants to complete their life cycle, at least as tested by a horizontal clinostat. Clinostatting does not prevent the completion of the plant life cycle. However, clinostatting does appear to slow down the reproductive process of Arabidopsis plants. Whether higher plants can continue to reproduce for many generations in a true micro-g environment of space can only be determined by long duration experiments in space.

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IMPORTANCE OF GRAVITY FOR PLANT GROWTH AND BEHAVIOR

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NASA Grants:
NGR 39-010-149 to U. of P.
NGR 39-030-010 to U.C.S.C.

RESEARCH EFFORTS -- Our principal research activities in the past year relate to seven experimental projects.

1. A study of hyponastic responses to incremental changes of an axially imposed centripetal force (2-axis clinostatting). A culture method was improved and validated for the production of dependably uniform test populations to be used in forthcoming experiments. (Research Task, EPIFOG)

2. Studies in support of Spacelab-1 Experiment, 1NS 101. Methods for preparing "soil" in flight hardware containers, colloquially termed "Pots", to ensure desired moisture content and miminal contamination probability were improved and validated prior to the SL-1 Mission. Both before and after the flight a tedious job of determining overall percision of measurement of the plant position coordinates on the image frames was carried out. Some flight data were used and also ground based data using flight hardware. The standard deviation of a large series of measurements was acquired and proved to be nearly the same for x and y coordinates. It amounted to ± 0.36 mm at the object plane, a number that was important for data analysis. (Research Task HEFLEX)

3. During development of Spacelab-4 Experiment 781236, which will use Avena seedlings for measurements of gravitropic responses of the coleoptile in microgravity to a range of g stimulus intensities delivered by programmed, laterally applied, centripetal forces the data yield will be greater insofar as subject response variation can be narrowed. Development of the grass seedling mesocotyl introduces a quantitatively important source of subject response variability. In some laboratories experimenters found that they could accept such reduction in precision of response measurements; in other laboratories the nature of the desired measurements made the larger response variability statistically unacceptable. In our case we began with the assumption that mesocotyl development would not be an unacceptable cause of data degredation. We were wrong.

Consequently we explored the mesocotyl growth pattern and established that, as predicated by "Avena lore", exposure to red light during early seedling ontogeny would suppress elongation of the first internode (usually referred to as mesocotyl) and would improve the uniformity of coleoptile development characteristics.

We designed a piece of flight hardware, crudely termed a mesocotyl supression box (MSB), to provide a standard red light irradiation of very young oat seedlings. We tested the efficacy of the MSB at various stages of seedling development. We are presently engaged in an even more precise determination of the minimal seedling age at which mesocotyl suppression can be effective because the answer has a significant impact on the proposed GTHRES experiment protocol and therefore is important in establishing "time line" requirements. (Research Task, GTHRES)

4. In the course of developement of GTHRES flight hardware and of hardware for the socalled Spacelab-4 (SLS-2?) FOTRAN Experiment, 781054, on which our colleague, Dr. D.G. Heathcote, is Principle Investigator, we designed, fabricated, and tested flight experiment type specimen housings -- referred to as "CUBES" (because they are) -- and we attempted, for obvious reasons, to make FOTRAN and GTHRES CUBES as nearly as possible identical even though the FOTRAN AND GTHRES Experiments are quite different in most respects. However, in our final designs, they are very similar but could not be made identical.

5. The choice of how many seedlings to accomodate in each CUBE is an important early decision that can be made only empirically or, if mathematically predictable, would have to be confirmed by experiment. With too many plants per cube optimal population size would not obtain. Our trial-and-error tests seem to be leading to a choice of 8 or 9 seedlings per CUBE. (Research Task, GTHRES)

6. Processing data from the Spacelab-1 Experiment proved to be more time consuming than we expected. Parly because about half the HEFLEX data was not readable and partly because all criteria for useful data could not be established in advance, we mounted a major effort (a) searching HEFLEX data for <u>all</u> episodes of unambiguous circumnutation recorded in microgravity, and (b) attempting to retrieve vibration and shock data recorded (or probably recorded) in flight and stored, along with about 2×10^{12} bits of other information in a vast repository which cannot be quickly accessed. Data reduction fortunately led to results that were not ambiguous. Many episodes of circumnutation were recorded on all but one of our test plants.

The question, how circumnutation could proceed in absence of a significant g force, remains unexplained; the flight experiment data which demonstrated that result leaves no doubt that the experiment was successful. This effort in support of data reduction and interpretation of a flight reinforces our confidence in the synergism between flight and ground based research. (Research Task, HEFLEX)

7. Clinostat Validation. It has been a long standing objective of our gound based efforts to test various plant response modes when exposed to physically different but putatively equivalent clinostat configurations. Unexpectedly, the Spacelab 1 HEFLEX Experiment results have made unique contributions to that effort. Only a small number of gravity related plant phenomena have been tested in clinostat simulated hypogravity and also in real hypogravity in space flight. Previous cases showed small nevertheless significant differences (which could easily be attributed to slightly imperfect simulation). The HEFLEX Experiment results showed not only that circumnutation occurred in microgravity but that the values for amplitude and period of the oscillations were intermediate between the values obtained at <u>1g</u> and at clinostat simulated zero <u>g</u>. In no other case has this been true. Circumnutation in space was <u>substantially more vigorous</u> than on the earth based clinostat! We should like to believe that clinostats can be reasonably good simulators of the hypogravity condition they imitate but the HEFLEX data did not bear this out. That result will be an important addition to our more general effort to test the validity of clinostat simulations. (Research Task, SIMCOM)

Other experimental research tasks were proposed to NASA. Ordinarily one might not consider the act of submitting a research proposal as a reportable event. However, when the amount of paper generated by that activity is measured in inches rather than millimeters, the effort was not insignificant. Two proposals for flight experiment development were submitted in February and October, 1984. (Research Tasks, AMYSED and GTRGMS)

SUPPORT OF SCIENTIFIC MEETINGS --Papers were presented at:
(1) Second European Symposium on Life Sciences Research in Space (sponsored by ESA and DFVLR); Porz-Wahn, Germany; 4-6 June 1984.
(2) International Forum on Spacelab and Space Station/Final Meeting of SL-1 IWG; Naples and Anacapri, Italy; 11-16 June 1984.
(3) XXV COSPAR Plenary Meeting; Graz, Aust.; 2-4 July 1984
(4) Annual Meeting, Amer. Soc. Plant Physiol; Davis, CA; 12-17 Aug 1984
(5) VI Annual Meeting, IUPS Commission on Gravitational Physiol.; Lausanne, Switzerland; 18-21 Sept. 1984

PROJECTED RESEARCH -- Grant supported studies will continue on research tasks, as mentioned above; EPIFOG, GTHRES/FOTRAN (only partly supported by grant), SIMCOM, and HEFLEX follow-on modeling of the drive and control mechanism for circumnutation.

RECENT PUBLICATIONS -- A preliminary report of some results of the HEFLEX flight experiment was published (1). A paper desribing tests of Helianthus plant culture in microgravity on STS-3 missions has been in press for some time and should appear soon (2). This is a more complete report of the HBT (HEFLEX Bioengineering Test) results which were presented briefly in an earlier paper (The Physiologist 25, N.6 Suppl: S 5-8, 1982). A paper, cited last year as in press, has been published (3).

(1) Brown, A. H. and Chapman, D. K. 1984. Circumnutation observed without a significant gravitational force in spaceflight. Science 225: 230-232.
(2) Brown, A. H. and Chapman, D. K. 1984. A test to verify the biocompatibility of a method for plant culture in a microgravity environment. Ann. Bot. 54: Suppl 3 "in press"
(3) Brown A. H. 1983. Resistence of nature Arabidopsis plants to mechanial deformation in relation to g-force during development. The Physiologist 26, No. 6 Suppl: S 149-150.

SESSION III: PHYSIOLOGICAL EFFECTS OF GRAVITY

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A. BIOLOGICAL SUPPORT STRUCTURES AND THE ROLE OF CALCIUM

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STRUCTURAL DEVELOPMENT AND GRAVITY

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RESEARCH PROJECTS

Our major research projects are designed to elucidate the mechanisms by which gravity loading and/or fluid distribution alter bone formation and/or resorption in rat bone. The research projects completed this year include: 1) analysis of bone parameters in rats from 6 weeks to 68 weeks of age, 2) restricted access area in which rats on the model were not allowed to touch any side of the cage, and 3) the effect of dietary calcium levels on bone formation and resorption rates in controls and head-down rats.

1) The major findings of this project, using Sprague-Dawley derived male rats, are a) the tibia and the vertebral column increase about 35% in length from 6 to 18 weeks of age, but elongate very little (about 5%) over the next 50 weeks; b) bone apposition rate at the tibiofibular junction (TFJ) decreases linearly from 9.4 microns/day at 6 weeks of age to about 1.9 μ/d at 14 weeks, 1.0 μ/d at 18 weeks, 0.4 μ/d at 28 weeks and 0.13 μ/d at 53 weeks of age; c) marrow area at the TFJ remains constant from 6 to 10 weeks, then increases from about 0.8 mm2 to 1.16 mm2 by 68 weeks of age suggesting that formation and resorption are comparable at this sampling site until 10 weeks of age when resorption predominates; d) body mass increases linearly from about 150gm at 4 weeks of age to 310gm by 8 weeks, 400 gm by 15 weeks, 550gm by 43 weeks, and 580 gm at 68 weeks of age.

2) Restricting the rats on the model so that they could not touch the plexiglas sides of the cage did not further decrease bone formation rates following 2 weeks of experimentation (controls = 0.053+/-0.013mm2/day, restricted = 0.038+/-0.008 mm2/day, suspended = 0.038+/-0.008mm2/day).

3) Marrow area in head-down rats was very similar to control rats; in both groups, marrow area was inversely related to dietary calcium suggesting that bone resorption at the tibial endosteum at the TFJ is more responsive to metabolic factors than to load-bearing. Bone formation at the periosteal surface of the tibia at the TFJ was not affected by diet in head-down animals while control rats showed a suppression of formation only at the very low level of dietary calcium (0.1% Ca, 0.3% P); bone formation in head-down rats was significantly lower than controls (40%) except in the very low Ca diet when the difference between groups was not significant. This experiment was done in collaboration with Drs. Bikle and Halloran.

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SHUTTLE PROJECT:

A significant portion of FY84 was spent as Project Scientist/Manager of a high school student shuttle project. The Weber Shuttle Student Involvement Project (SSIP) hypothesized that the development of adjuvant induced arthritis had a gravity component. Studies on an animal model simulating some aspects of spaceflight (unloading of rear limbs, fluid shift toward the head) suggested that unloading of the limbs and/or fluid shifts inhibited the systemic portion of the disease. The experiment flew on STS41-B. Data collected immediately postflight suggested that gravity did not contribute to the development of the arthritic process. However, immunologically different animals had been used for the preflight data base; gnotobiotic animals were used for flight and specific pathogen free (SPF) animals had been used for all ground based studies. The flight experiment was based on a time course of the disease process found in SPF animals (about 10 days for apparent systemic disease), whereas the onset of systemic disease required about 14 days in the gnotobiotic rats. Reentry at the time the systemic disease occurred may have significantly impacted the data. These data make a very strong case for requiring that all data collected prior to flight be done on exactly the same type of rat as well as the same strain. The difference in disease time course was unanticipated prior to flight.

The normal control rats aboard STS-41B ate more food and gained more weight than the ground controls. However, analysis of the data suggested that both groups were adding body mass at the same rate when expressed as g gained/kcal food consumed. The rats were housed in colony type cages with 3 rats/cage; prior Cosmos studies where rats were housed in small individual cylinders suggested that rats in space gained less weight per gram of food consumed. Thus, the Cosmos rats may have been slightly stressed leading to a decreased body mass gain. If flight rats gain at the same rate as ground controls, then pair-feeding the ground control animals to the food consumption of the flight rats will not be necessary.

Following the Weber experiment, a water bottle designed for the Animal Enclosure Module (AEM) by Jack Sweeney of Orthopedic Hospital, Los Angeles and fabricated by General Dynamics was tested in our laboratory. The water bottle which is spring-loaded and, hence, gravity independent, holds approximately 1500 ml. Four adult (550g) Long-Evans rats were placed in the AEM and 4 were kept in colony cages (2/cage). Over a 9 day period, both groups drank about 35 ml tap water/ rat/day and ate about 25 gm rat chow/rat/day. Based on this information, the water bottle should supply similar rats approximately 11 days of water.

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The Role of Vitamin D in the Bone Changes Associated with Simulated Weightlessness

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NASA Grant NAGW-349

Normal mineral metabolism is disrupted during space flight. Urinary excretion of Ca increases and the skeleton becomes osteopenic. These metabolic abnormalities appear to be induced by the weightlessness experienced during space flight. In order to study this phenomena we have made use of the tail suspended rat model to simulate weightlessness. In this model rats are suspended by their tails such that the hind limbs are not allowed to touch the ground (are unloaded) while the forelimbs remain weight bearing (are loaded). Using this model, we have demonstrated that unweighting of the rear limbs results in a transitory reduction in bone formation in the unweighted limbs (e.g. tibia) but not in the weighted limbs (e.g. humerus) as evidenced by a reduction in ^{45}Ca and ^{3}H-proline uptake by the bone and a reduction in total bone mineral. This transitory reduction (days 2-7 of suspension) is followed (days 8-15 of suspension) by a trend toward normalization of bone formation. The question arises as to the role of vitamin D in these changes in bone metabolism. To examine this issue we measured the serum concentrations of $1,25(OH)_2D$ and $24,25(OH)_2D$ in rats sacrificed after 2, 5, 7, 10, 12 and 15 days of suspension. The serum concentration of $1,25(OH)_2D$ decreased by 48% after 2 days of suspension and by 60% after 5 days of suspension. Between days 5 and 15 of suspension, the concentration of $1,25(OH)_2D$ in the serum gradually increased from its nadir at day 5 reaching a level not significantly different from the pair-fed control animals by day 15. The serum concentration of $24,25(OH)_2D$ increased (but not significantly) between days 1 and 5 of suspension and then gradually decreased towards normal between days 5 and 15. The time course of the changes in the circulating concentrations of $1,25(OH)_2D$ and $24,25(OH)_2D$ mirror almost precisely the changes in bone metabolism. This raises the question as to the relationship between the changes in vitamin D metabolism and bone metabolism. Are the bone changes due to the change in serum concentration of $1,25(OH)_2D$ or are the changes in bone formation (viz. a reduction in Ca uptake) causing a reduction in Ca flux out of the serum pool and thereby suppressing $1,25(OH)_2D$ production. To answer this question we infused suspended and pair-fed rats with $1,25(OH)_2D$ or vehicle at a constant rate for 2 weeks using Alza osmotic minipumps. The rationale was to maintain a constant high level of $1,25(OH)_2D$ in the serum throughout the period of suspension and then determine if, under these conditions, bone formation was still impaired. Animals

were sacrificed at 2, 5, 8 and 12 days of suspension. Suspended animals infused with vehicle exhibited the expected fall in serum $1,25(OH)_2D$ between days 2 and 5 of suspension and the return toward normal between days 5 and 12. In contrast, all animals, i.e. both suspended and pair-fed control, infused with $1,25(OH)_2D$ had essentially the same serum concentration of $1,25(OH)_2D$ (200 pg/ml). As expected, suspension had no effect on hormone concentration in the $1,25(OH)_2D$ infused animals. Nevertheless, both vehicle and $1,25(OH)_2D$ infused suspended rats exhibited the same reduction in bone mineral, and uptake of ^{45}Ca. These results suggest that the transitory reduction in circulating $1,25(OH)_2D$ during suspension is not likely to cause the abnormalities in bone metabolism but rather that the changes in bone metabolism are primary and cause the fall in serum $1,25(OH)_2D$ concentration. These observations further support the hypothesis that the metabolic abnormalities in bone associated with simulated weightlessness are due to the direct effect of unweighting on the bone.

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THE SALUTARY EFFECT OF DIETARY CALCIUM ON BONE MASS IN A RAT MODEL OF SIMULATED WEIGHTLESSNESS

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NASA Grant NAGW-236

In previous studies we have observed that unweighting the hind limbs of a rat while preserving normal weight bearing on the fore limbs results in a decline in bone mass in the unweighted limbs in comparison to pair-fed controls. In this study we asked two complementary questions:

1) Could dietary calcium supplementation reduce the differences in bone mass of unweighted limbs and normally weighted limbs?
2) Do parathyroid hormone (PTH) and 1,25-dihydroxyvitamin D ($1,25(OH)_2D$) respond differently to dietary calcium in unweighted animals in comparison with pair-fed controls?

To answer these questions we unweighted the hind limbs of rats by a tail suspension method while feeding them diets containing 0.1% to 2.4% calcium. After 2 weeks we measured serum calcium, phosphorus, PTH, and $1,25(OH)_2D$. We also determined intestinal calcium transport by the everted gut sac method and measured bone mass, ash weight, and calcium in the tibia, L-1 vertebra, and humerus. No significant differences in body weights were observed among the various groups. Suspended rats maintained constant levels of serum calcium and phosphate over the wide range of dietary calcium. Serum PTH and $1,25(OH)_2D$ and intestinal calcium transport (measured in vitro) fell as dietary calcium was increased. With the increase in dietary calcium, bone calcium in the tibia, vertebra and humerus increased 60%, 85%, and 50%, respectively, in both suspended animals and pair-fed controls. Bone calcium in the tibia and vertebra from suspended rats remained less than that from pair-fed control. These data suggest that although no striking difference between suspended and control animals was observed in response to dietary calcium, increasing dietary calcium may reduce the negative impact of unloading on the calcium content of the unweighted bones. The salutary effect of high dietary calcium appears to be due to inhibition of bone resorption rather than to stimulation of bone formation.

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Title: Actual and Simulated Weightlessness Inhibit Osteogenesis in Long Bone Metaphysis By Different Mechanisms

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Introduction: Weightlessness and simulated weightlessness (head down suspension) inhibit the rate of periosteal bone formation in long bones (1-3). Formation of preosteoblasts is suppressed in periodontal ligament (PDL) of maxillary molars (4), suggesting a generalized block in osteoblast histogenesis. Growth in length of long bones is decreased by simulated weightlessness (2), but there are no reliable data on the influence of actual weightlessness on metaphyseal growth. As an index of osteogenic activity this study utilizes the nuclear size assay for assessing relative numbers of osteoblast precursor cells (5) in the primary spongiosa of growing long bones subjected to actual and simulated weightlessness.

Methods: Four groups of five 83 day old male Wistar rats were prepared as follows: 1. 18.5 days of spaceflight aboard Cosmos 1129, 2. ground based flight controls for Cosmos 1129, 3. simulated weightlessness for 21 days by 30° head down suspension via orthopedic traction on the tail (3), and 4. unsuspended controls. The primary spongiosa of proximal metaphysis of the tibia was studied for suspended animals. The same area from Cosmos 1129 animals was sampled from the ulna because that was the only long bone available. Specimens were demineralized, embedded in plastic and sectioned at 3 μm in the frontal plane. According to nuclear volume measured at 1000X, 100 fibroblast-like cells/animal were classified as: A/A'(< 80), B(80-119), C(120-169), D(≥ 170) and C/D combined ($> 120\ \mu m^3$).

Results: Comparing suspended animals to unsuspended controls, tibial primary spongiosa cell distributions increased in A/A' cells (45.8 ± 3.7 vs 36.0 ± 1.6, $p < 0.05$) but decreased in D(10.8 ± 1.8 vs 16.8 ± 1.0, $p < 0.02$) and C/D cells (26.4 ± 2.3 vs 34.0 ± 1.9, $p < 0.05$). There was no difference in total number of osteogenic cells/mm bone surface (76.5 ± 5.5 vs 75.9 ± 11.4). Comparing Cosmos 1129 animals to flight controls, ulna primary spongiosa cell distributions also increased in A/A' cells (66.8 ± 5.7 vs 50.4 ± 3.6, $p < 0.05$) and decreased in C(6.8 ± 1.3 vs 15.0 ± 2.1, $p < 0.01$) and C/D cells (9.8 ± 3.0

vs 22.0 ± 4.0, $p < 0.05$). There was a decrease in total number osteogenic cells/mm of growth plate surface (30.2 ± 3.2 vs 58.6 ± 6.8, $p < 0.01$).

Discussion: Previous cell kinetic studies have demonstrated: 1. A cells are self perpetuating less differentiated precursors, 2. A' cells, a subset of A cells, are kinetically distinguishable, committed osteoprogenitors, 3. B cells are non-osteogenic, 4. C cells are G_1 stage preosteoblasts, 5. D cells are G_2 stage preosteoblasts, and 6. the osteoblast (Ob) differentiation sequence is A→A'→C→D→Ob. The A'→C upward shift in nuclear volume is a key, rate limiting step in Ob production. A block at this step results in an accumulation of A/A' cells and depletion of C/D cells. Since this pattern was observed in both instances, the A'→C shift in nuclear size, which is a morphological manifestation of change in genomic expression (6), is inhibited in both actual and simulated weightlessness. However, the decrease in total number osteogenic cells seen only in Cosmos animals and previously observed in their PDL (4), may be a specific inhibition of cell proliferation. It appears that head down suspension only partially simulates the effects of weightlessness on osteogenic cells.

Conclusions: 1. Actual weightlessness decreases total number of osteogenic cells and inhibits differentiation of osteoblast precursor cells, 2. Simulated weightlessness suppresses only osteoblast differentation, and 3. The nuclear morphometric assay is an effective means of assessing osteogenic activity in the growing metaphysis of long bones.

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N85-26075

Title: Quantitative Alterations in the Function of Bone Forming Cells Due to Reduced Weight Bearing.

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Introduction: Rats subjected to spaceflight or suspended in a non-weight bearing position for 2-3 weeks, show a significant reduction in new bone formation (Morey and Baylink,1978; Morey, 1979). We have previously suggested, on the basis of morphology and histochemistry, that this reduction is associated with a decrease in alkaline phosphatase activity in the differentiated osteoblast population. Those cells in the diaphyseal region of bone are more affected than the same cell type in metaphyseal bone. These studies are being continued on a more quantitative basis, with measurements of alkaline phosphatase activity in specific regions of bone, and the autoradiographic localization of H^3-proline in bone forming areas. Concomitant with decreased bone matrix synthesis, the osteoblast population also demonstrates changes in the Golgi/lysosomal complex as a result of whole animal suspension. Morphometric techniques are being applied for quantitation of the lysosomal population and the percentage of lysosomal or Golgi bodies containing acid phosphatase activity.

Methods: Mature male rats, 250-300 grams, were suspended for 7-21 days at the NASA-Ames Research Center. Femurs and/or tibias were collected at the end of the experimental period and placed into (a) cold 2% glutaraldehyde in 0.1M cacodylate buffer,pH 7.4, for four hours, or (b) cold 2% para-formaldehyde and 0.5% glutaraldehyde in 0.1M cacodylate buffer, pH 7.4, for 18 hours. These tissues were decalcified prior to autoradiography or histochemistry. For quantitative data, frozen sections of tissue were obtained and measured for alkaline phosphatase activity using a fluorescent method with methyl umbelliferyl phosphate as a substrate at pH 10.0. Cell counts or surface area of bone covered with reactive osteoblats were determined on alternate sections not used for the alkaline

phosphatase measurements. Autoradiographic localization of H^3-proline incorporation into new collagen matrix was accomplished on similar frozen sections. For morphometric determination of lysosomal activity, sections of aldehyde-fixed decalcified tissues were incubated for acid glycerophosphatase activity, pH 5.0, and prepared for electron microscopy. Random blocks of tissues were chosen for study and thin sections photographed at random. Photographs at 45,000 final magnification were covered with a grid of 100 or 400 points and volume densities of cytoplasmic organelles were determined by stereological methods (Weibel,1973).

Results: Alkaline phosphatase activity within diaphyseal osteoblasts did not show a statistical change within the first five days of suspension. A reduction in alkaline phosphatase activity, reduced H^3-proline incorporation into new matrix, and increased lysosomal activity within osteoblasts, were found by twelve days of suspension. Such results, which initially appear unrelated, could be explained by suggesting a change in the cytoskeletal component of the osteoblast. For example, previous studies (Doty,1980) have shown that in vitro colchimide administration, which inhibited microtubule formation, reduced osteoblastic alkaline phosphatase activity within 24 hours.

We have begun to localize actin within osteoblasts using immunocytochemistry and presently are comparing actin distribution at the fifth day and twelfth day of suspension. The cytoskeletal attachment to the cell membrane or at the membrane/matrix interface could be the cellular "sensing" device for detecting change in gravitational forces. The inter-relationships between the various components of the cytoskeletal system could then affect the metabolic activities of the osteoblasts population.

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New Techniques for Studying Calcium Gradients

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NASA Grant NAGW-515

The aspects of our current research most relevant to space and gravitational biology have to do with improved techniques for detecting and manipulating spatial gradients of cytosolic free Ca^{2+} concentrations $[Ca^{2+}]_i$ and intracellular stores. Three areas of recent progress are: 1) development of new fluorescent indicators for Ca^{2+} that are the first to be suitable for measuring $[Ca^{2+}]_i$ and its inhomogeneities i individual cells; 2) invention of photolabile chelators which shift irreversibly from high to low affinity for Ca^{2+} upon illumination, permitting light-controlled jumps in $[Ca^{2+}]_i$; 3) new fixation methods to trap dynamic intraorganellar Ca stores in a form readily visible by electron microscopy.

The new group of indicators comprises what we believe to be the best dyes now available for measurement of $[Ca^{2+}]_i$ in most intact cells. The most attractive members of this family, "fura-2" and "indo-1", have 3 main and 2 minor advantages over their predecessor "quin2". 1) Molecule for molecule the new dyes are about thirty times more fluorescent than quin2, an improvement due to a six-fold increase in extinction coefficient and about five-fold enhancement in quantum efficiency. This huge increase in brightness can be used either to reduce dye loading, thus minimizing exogenous buffering of $[Ca^{2+}]_i$ and the possibility of toxic side effects, or to make signal detection feasible from single cells, as will be shown below. 2) Both the Ca-free dyes and their Ca complexes are highly fluorescent but at differen wavelengths. Fura-2 changes mainly its peak excitation wavelength with Ca^{2+}, while indo-1 shifts both its excitation and emission maxima. Detecting Ca^{2+} by means of the ratio between intensities at two wavelengths is far more stable and reliable than relying on intensity at just one wavelength as was necessary with quin2. 3) Fura-2 and indo-1 can be used with wavelengths of 350 nm and above. While this may seem a small difference from the 339 nm excitation for quin2, those few nm make possible the use of this dye in conventional microscopes with glass optics and in flow cytometry systems using argon or krypton lasers. 4) The new dyes have about two-fold greater effective dissociation constants for Ca^{2+} than quin2 has. Resolution of $[Ca^{2+}]_i$ level near or above 10^{-6} M is therefore improved. 5) The new dyes have more selectivity than quin2 has for Ca^{2+} over competing divalents such as Mg^{2+}, Mn^{2+}, and Zn^{2+}. The improvements are factors of 4, 12, and 40 respectively. It should be noted that the syntheses are not trivial: to make the intracellularly hydrolyzable esters of fura-2 and indo-1 from commercially available chemicals requires fifteen and thirteen steps respectively.

With fura-2 we have collaborated with Dr. R.A. Steinhardt to make the first measurements of $[Ca^{2+}]_i$ during the complete mitotic cycle of individual sea urchin zygotes. They show not only the known large spike of $[Ca^{2+}]_i$ at fertilization but also a later elevated pedestal level from which rise smaller blips of $[Ca^{2+}]_i$ during pronuclear movement, nuclear-envelope breakdown, the metaphase-anaphase transition, and cytokinesis. Fura-2 has also shown the $[Ca^{2+}]_i$ transients associated with contraction of single smooth muscle cells (done with Dr. F.S. Fay) and with lectin stimulation of single thymocytes. With indo-1 it is now possible to analyze the population distribution and heterogeneity of $[Ca^{2+}]_i$ using flow cytometry.

Another class of new Ca^{2+} chelators uses light not to signal $[Ca^{2+}]_i$ but to release Ca^{2+}. These molecules are the first realizations of the much sought after "caged calcium." The structure and mode of operation of the best present version,

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nitr-2, will be discussed. Before photolysis nitr-2 binds Ca^{2+} with an effective dissociation constant near 170nM; afterwards, the binding weakens to a K_d of 7 μM. Prof. R.S. Zucker has microinjected nitr-2 into Aplysia neurons and verified that illumination can now trigger membrane currents already known to be Ca^{2+}-activated. However, nitr-2 could use considerable improvement in extinction coefficient, quantum efficiency of photolysis, pre-illumination affinity for Ca^{2+}, and speed of Ca^{2+} release (currently ~0.4 sec exponential time constant).

A completely different approach to studying calcium movements became available when Martin Poenie joined the PI's laboratory. During his Ph.D. work under Prof. David Epel at Stanford, Dr. Poenie had devised a group of remarkable new techniques for histological fixation of calcium precipitates. Traditional methods have used either oxalate or antimonate to try to trap organelle-sequestered Ca^{2+} as electron dense precipitates. Oxalate tends to have insufficient precipitating power, whereas antimonate tends to deposit precipitate indiscriminately. The Poenie methods rely on fluoride, which seems to penetrate cells and organelles more quickly and to form a more insoluble and stable precipitate with Ca^{2+}. These techniques applied to sea urchin eggs successfully revealed calcium stores shown by electron microscopy to reside in tubular reticular organelles. Upon fertilization, these calcium stores seemed to discharge, but refilled several minutes later. Other discharges of Ca^{2+} stores were analogously seen in sea urchin sperm during fertilization and in scorpion tail muscle during contraction. In my lab Dr. Poenie has collaborated with Prof. Beth Burnside to look at sunfish retina. In dark adapted rods there is good preservation of calcium deposits specifically inside the disks, not in the cytosol, of rod outer segments. Rods momentarily exposed to light are found to have lost most of their EM-visible intradiskal calcium, in agreement with the Yoshikami-Hagins hypothesis of visual transduction. These fixation methods appear highly promising as a way to follow dynamic localized changes in intraorganellar calcium stores.

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Role of Glucocorticoids in the Response to Unloading of Muscle Protein and Amino Acid Metabolism

Marc E. Tischler and Stephen R. Jaspers

NAGW-227

Intact control (weight bearing) and suspended rats gained weight at a similar rate during the 6 day period following tail-casting (26 ± 2 and 21 ± 3 grams, respectively). Adrenalectomized (adx) weight bearing rats gained less weight during this period (18 ± 1 grams) while adrenalectomized suspended rats showed no significant weight gain (3 ± 2 grams). Cortisol treatment of both of these groups of animals caused a loss of body weight (16 ± 1 and 17 ± 1 grams, respectively).

The slower growth rate of adx weight bearing rats was reflected in little growth of the extensor digitorum longus (EDL) muscle and no significant growth of the soleus muscle. In the adx suspended rats, the EDL failed to grow and the soleus atrophied to a greater extent. Both muscles showed a loss of mass in the cortisol treated rats. These results show that adrenalectomy caused stress in both weight bearing and suspended rats but had a greater effect in the suspended group. This effect was enhanced by administration of 2 mg/100 g body wt of cortisol acetate.

Comparison of protein metabolism in soleus muscles of weight bearing and suspended rats had shown previously that the atrophy was due to both slower protein synthesis and faster protein degradation. This difference in protein synthesis was abolished in adx animals and not restored by administration of the same dose of cortisol, which slowed this process in muscles of both groups of adx rats. The difference in protein degradation was only partially lowered by adx. Administration of cortisol at the same dose to both groups increased this difference from 0.21 nmol tyrosine/mg protein/2 h to 0.53 nmol tyrosine/mg protein/2 h. In the EDL muscle cortisol slowed protein synthesis to the same extent in both groups. Cortisol lowered protein degradation in the EDL muscle of weight bearing animals but had no effect in suspended rats. These results support previous findings that in fed adx rats, this cortisol dose slows protein synthesis and either has no effect or diminishes protein degradation. Furthermore, these findings suggest that the slower protein synthesis and, in part, the faster proteolysis, of the unloaded soleus muscle may be due to higher levels of circulating glucocorticoids in the suspended animals. However, part of the response of the protein degradation must be unrelated to steroid levels.

Amino acid metabolism was also studied in these animals. Previous work showed a slower synthesis of glutamine by soleus muscles of suspended animals despite greater activity of glutamine synthetase. This slower synthesis was reflected in lower ratios of muscle glutamine/glutamate in the unloaded (1.42) than in the weight bearing (2.68)

soleus. Adrenalectomy had no significant effect on this ratio in the soleus. In contrast, adx lowered this ratio by 36-40% in the EDL and cortisol treatment restored this ratio to normal. In the soleus, cortisol treatment increased the ratio in the weight bearing, but not in the unloaded soleus. The lack of response in the unloaded muscle probably is due to limiting amounts of tissue ammonia, as noted previously.

Since muscle glutamine synthetase activity is very sensitive to alterations of steroid levels, it was measured in these muscles. Adx resulted in lower activity in weight bearing and unloaded soleus (75 and 94% respectively) and EDL (86-90%) muscles. In the weight bearing muscles, cortisol (2 mg/100 g body wt/day) restored these activities to 81-83% of normal. After cortisol treatment, the activity in the unloaded EDL was similar to the control muscle but much lower (-54%) than in the EDL of intact suspended rats. This finding suggested that the higher activity of glutamine synthetase in EDL muscles of control and suspended rats was probably due to higher circulating glucocorticoids. In the soleus muscle, despite administration of the same cortisol dose to adx weight bearing and suspended rats, the enzyme activity was 59% greater in the unloaded muscle. This finding suggested a greater sensitivity of this muscle to glucocorticoids, as suggested by an earlier observation of increased numbers of receptors after unloading (Steffen and Musacchia, The Physiologist 25 (suppl.) S151-S152 1982). In muscle, branched-chain amino acids serve as the primary donor of α-amino groups for glutamate, and hence for alanine and glutamine production. In soleus or EDL muscles, unloading lead to faster degradation of leucine, isoleucine and valine. Although adx abolished this difference in the EDL muscles, it had no effect in the soleus. Cortisol administration increased degradation of leucine to the same extent in EDL muscles of weight bearing and suspended animals. Therefore, higher circulating glucocorticoids in suspended rats may account for the difference in branched-chain amino acid degradation in this muscle. In contrast, the soleus of adx suspended rats was more responsive to cortisol administration (as noted for glutamine synthetase). While greater numbers of receptors may be important in the unloaded soleus, the data for untreated adx rats suggest that another factor may be important, as well, for the increased leucine degradation following unloading of the soleus muscle.

In summary, these results show several important findings: 1) Metabolic changes in the EDL muscle of suspended rats are due primarily to increased circulating glucocorticoids. 2) Metabolic changes in the soleus due to higher steroid levels are probably potentiated by greater numbers of receptors. 3) Not all metabolic responses in the unloaded soleus muscle are due to direct action of elevated glucocorticoids or increased sensitivity to these hormones.

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Constant fiber number during skeletal muscle atrophy and modified arachidonate metabolism during hypertrophy.

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A previously documented shift from Type I to IIA predominance of the soleus muscle during rat suspension was further investigated to determine if this shift was by selective reduction of a single fiber type, simultaneous reduction and formation of fibers with different fiber types, or a transformation of fiber type by individual fibers. By partial acid digestion and dissection, average total soleus fiber number was found to be 3022 ± 80 (SE) and 3008 ± 64 before and after four-week suspension (n=12). Fiber number of muscles excised before suspension was linearly related to that of contralateral muscles after suspension (r=0.9). From myosin ATPase- and NADH diaphorase-stained cross-sections, fiber area declined significantly in Type I fibers after two weeks suspension, but remained unchanged in Type II fibers after four weeks. Type I/II percentages of fiber number changed from (82/18) in control muscles to (45/53) after four weeks suspension. These data decrease the probability of selective Type I reduction, and previous data showing no significant degenerating/regenerating fibers decrease the probability of simultaneous reduction and formation of different fiber types. Taken together these data suggest the possibility of transformation of individual fibers from characteristics of a larger, but atrophying, Type I fiber into those of the smaller Type IIA fiber.

Another area of current research was based on previous studies which indicate that prostaglandins are biosynthesized by skeletal muscle and evoke protein synthesis and degradation. Accordingly, indomethacin, a cyclo-oxygenase inhibitor, was administered to rats during recovery from suspension while the soleus muscles are normally undergoing marked hypertrophy. Indomethacin (0.7 mg/day) reduced increases in rat soleus weight by 50% and Type I fiber hypertrophy by 41% during a seven-day recovery from rat suspension for two weeks.

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BLOOD PRESSURE RESPONSES AND MINERALOCORTICOID LEVELS IN THE SUSPENDED RAT MODEL FOR WEIGHTLESSNESS.

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Cardiovascular (CV) responses and fluid/electrolyte shifts seen during space flight have been attributed to cephalad redistribution of vascular fluid. The antiorthostatic (AO) rat (suspended head-down tilted, 15-20°) is used to model these responses.

Current studies show that elevated blood pressures in AO rats are sustained for periods up to seven days. Comparisons are made with presuspension rats. Increased blood pressure in head down tilted subjects suggests a specific response to AO positioning, potentially relatable to cephalad fluid shift. The blood pressure results are presented in Table 1. To assess a role for hormonal regulation of sodium excretion, serum aldosterone levels were measured. Circulating aldosterone levels were seen to increase (about 100%) during seven days of AC suspension. Sodium excretion also increased significantly during AO suspension and potassium excretion increased during the last day. The results of serum aldosterone concentration and sodium and potassium excretion are presented in Table 2. These results suggest that aldosterone may not be involved in the long term regulation of increased sodium excretion in AO rats. These studies continue to show their prospective usefullness as models for the development of animal protocols for space flight. Supported by NASA Grant NSG 2325.

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TABLE 1. Blood Pressures in mmHg: (mean arterial = MAP, diastolic = D, systolic = S, pulse = PP) and heart rate (HR = bpm) in head-down tilted rats (15-20°).

	0	1	3	7	R1
MAP	102±2 (15)	109±13 (6)	114±5 (5)	121±5 (8)	102±2 (4)
D	88±2 (15)	95±5 (6)	101±6 (5)	107±5 (8)	86±2 (4)
S	130±2 (15)	137±4 (6)	145±6 (5)	149±17 (8)	134±6 (4)
PP	41±2 (15)	42±2 (6)	43±6 (5)	46±2 (8)	42±4 (3)
HR	423±11 (14)	460±13 (6)	457±14 (5)	451±9 (8)	471±8 (4)

0 = Pre suspension
1, 3 and 7 = days of suspension
R1 = 1 day recovery

Data are: mean ± SEM and numbers of rats (n)

TABLE 2. Serum aldosterone (pg/ml) and urinary excretion of Na^+ and K^+ (mEq/100g/d) in metabolism cage control (MCC) and antiorthostatic (AO) suspended rats.

Group	Aldosterone	Na^+	K^+
Day 0 MCC	78.4±30.3 (12)	0.85±0.06 (9)	1.62±0.09 (9)
Day 1 AO	157.1±35.6 (12)	0.83±0.10 (8)	1.31±0.09 (11)
Day 3 AO	115.5±22.2 (5)	1.09±0.13 (6)	1.77±0.18 (6)
Day 7 AO	163.6±51.7 (9)	1.30±0.08 (8)	2.15±0.16 (8)

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Ninth Annual Space Biology Symposium, Harpers Ferry, West Virginia
November 6-9, 1984

N85-26080

EFFECTS OF MUSCLE ATROPHY ON MOTOR CONTROL: CAGE-SIZE EFFECTS

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NASA Grant NAGW-338

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On the basis of preliminary observations (Stuart and Enoka, 1982; Enoka and Stuart, 1983), we have suspected that the small-cage-reared rat might be an inappropriate model for the study of altered-activity effects. To address this issue, we have raised two populations of male Sprague-Dawley rats, one group in the conventional minimum-specification cages (46-49 x 25-28 x 20 cm; 4-7/cage) and the other group in a much larger cage (320 x 183 x 100 cm; 15/cage). When the animals were mature (125-150 d), we compared the physiological status of the soleus (SOL) and extensor digitorum longus (EDL) muscles of the small- and large-cage animals. These particular test muscles were chosen as representative of slow- (SOL: fiber-type distribution, 84% SO, 16% FOG) and fast-contracting (EDL: 3% SO, 59% FOG, 38% FG) muscles, respectively. The comparison was accomplished by subjecting the test muscles to the experimental protocol depicted in Figure 1.

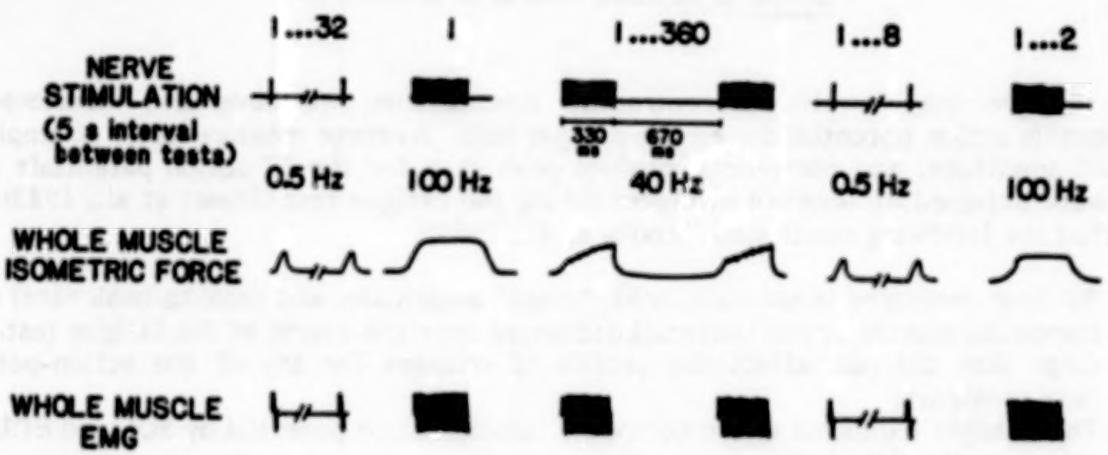

Figure 1. The experimental protocol which involved supramaximal intermittent stimulation of the nerve to the test muscle and the measurement of the associated compound muscle action potential and the force exerted by muscle.

The analyses have focused on a comparison of whole-muscle properties including the performance of the test muscle during a standardized fatigue test in which the nerve to the test muscle was subjected to supramaximal intermittent stimulation (trains of 13 pulses at 40 Hz repeated every one second for six minutes; Burke et al., 1971). Cage-size effects included (Table 1; Rankin et al., 1984):

1. A more rapid acquisition of adult physical characteristics by the large-cage rats.
2. A greater body weight but lesser EDL normalized muscle weight for the large-cage animals.
3. The test muscles in the large-cage-reared rats were significantly more fatigue resistant.
4. More rapid post-tetanic relaxation in the large-cage muscles.

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Since fatigue may involve precontractile mechanisms, we have also monitored the compound muscle action potential during the fatigue test. Average measurements of amplitude, area, "mean" amplitude, and normalized peak-to-peak rate for the 13 action potentials within each train were obtained at selected instances during the fatigue test (Stuart et al., 1983). The data supported the following conclusions (Enoka et al., 1984):

1. All four measures (amplitude, area, "mean" amplitude, and peak-to-peak rate) of the compound muscle action potential decreased over the course of the fatigue test.
2. Cage size did not affect the profile of changes for any of the action-potential measurements.
3. The changes exhibited in the compound muscle action potential by SOL and EDL were substantially different.
4. Except for SOL of the large-cage rats, there was a high correlation between all four measures of the compound muscle action potential and the peak tetanic force during the fatigue test. That is, except for large-cage SOL, either the electrical activity largely determines the force profile during the fatigue test or else contractile-related activity substantially affects the compound muscle action potential.

Taken together, these data suggest that the small-cage-reared male rat is a model of reduced activity. Therefore, we conclude that the large-cage-reared rat is a more appropriate control for the study of the effects of reduced activity. However, preliminary results from a second series of studies in which female rats were used suggest that cage size may be of lesser significance in studies using female rats.

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Disuse Induced Changes in the Cholinergic System of Sciatic Nerve and Slow and Fast Twitch Muscle of Rat

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NASA Grant NAG 2-301

There have so far been no reports available studying the cholinergic system of nerve and muscle such as choline acetyltransferase (ChAT) and acetylcholinesterase (AChE) and the acetylcholine receptor (AChR) during suspension induced hypokinetic conditions. The key enzymes involved in the synthesis and hydrolysis of the neurotransmitter, acetylcholine (ACh), ChAT and AChE are reported to be synthesized in the cell bodies of cholinergic motor nerves which innervate skeletal muscles. Following synthesis both these enzymes are transported by axoplasmic flow to the nerve terminals. ChAT is thought to remain highly localized in nerve terminals with low concentration in the muscles proper, whereas AChE is present in the axon and its activity is equally high in SOL and higher in EDL. Its presence in mature muscle depends to a large extent on innervation and muscle activity.

Among various reported models for studying disuse and stimulation of weightlessness, we have chosen hindlimb suspension as a model of disuse to evaluate the effects of decreased activity on muscle characteristics.

Experiments were initiated to study the effects of reduced muscle activity on AChE and its molecular forms, choline acetyltransferase and nicotinic receptor binding in innervated slow and fast muscle.

The weight of SOL was reduced to 64% within one week and continued to decrease progressively up to the third week when the weight was reduced to 40% as compared to controls. EDL showed a significant decrease in its weight only at the end of three weeks hypokinesia when it was reduced to 71% of control.

Biochemical Studies: Hypokinesia caused a significant increase in ChAT activity in both sciatic nerve and in hindlimb muscles (SOL and EDL) when results were expressed either as nmol ACh/mg protein/h or umol ACh/g tissue/h.

Recovery was complete within one week after removal of suspension in nerve and muscles.

In hypokinetic animals, EDL-AChE did not show a significant change when activity was calculated per g muscle or per mg protein, however, AChE activity in soleus increased by 161% and 261% when calculated on the basis of per g muscle or per mg protein, respectively.

When individual molecular forms of AChE were assayed an increase of all four major forms: 16S, 12S, 10S and 4S were found in SOl whereas, in the EDL, no significant change was observed in the 4S and 16S while the 10S was increased. AChE activity in sciatic nerve was not affected significantly up to three weeks of hypokinesia from that of controls.

Data on nicotinic acetylcholine receptor binding using ^{3}H-ACh as ligand indicate that two weeks hypokinesia caused a twofold increase ($p < 0.05$) in receptor binding in solubilized membrane preparations prepared from both EDL and SOL compared to control.

Histochemical Studies: The cross sectional area of SOL and EDL were significantly reduced when sections were made at mid length through the muscle belly. The type I fiber of the SOL was most affected by atrophy, while the same type in the EDL was less reduced in size. This was also true for the other fiber types. The fiber type distribution showed also changes with disuse.

In SOL, the proportion of fibers staining as type I fibers was reduced from 78% in control to 55%, while IIa fibers proportion increased from 2% to 20% and IIb fibers from 20% to 25% of total fibers population. In EDL, a muscle that has very few type I fibers to begin with, their presence was still further reduced. The slight change was seen in the number of type IIa fibers which increased from 40% to 45%, while the majority of the fibers (56%) were type IIb which showed no significant change in the % distribution of total fiber types. Since in none of these sections, whether from EDL or SOL, were there any signs of fiber splitting, denervation or degenerating fibers; the above described alterations most likely are changes in reaction characteristics within individual fibers.

While disuse affected mainly AChE of SOL with little change in the EDL enzyme, its effect on ACh binding seemed to be similar in SOL and EDL. Whether this increase in receptors reflects changes in non-endplate regions only remains to be seen. Experiments are in progress to study these changes in endplate and non-endplate regions separately.

It is evident from the present morphological findings and the biochemical data that some properties of skeletal muscles are strongly dependent on patterns and level of loadbearing and on motor unit activity. With suspension induced disuse the usually slow SOL appeared to change its characteristics such as fiber type distribution and AChE activity to one that more resembled a faster muscle.

It is important to note that hypokinesia induced changes either physiological, biochemical or morphological, are totally reversible as the induced changes returned to control levels within a week after cessation of disuse.

B. GRAVITY SENSITIVE SYSTEMS - MECHANISMS AND RESPONSES

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THERMOREGULATORY AND VESTIBULAR SYSTEMS IN THE RAT

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NSG-2234

Introduction The effects of decreasing brain temperature upon the transmission of neural signals along the brainstem auditory pathway has been well documented in cats and mice. The increase in the interpeak latencies of components of the brainstem auditory evoked response (BAER) has indicated that a progressive slowing occurs along the pathway as the signals ascend toward higher brainstem areas.

In comparison to the numerous studies on the auditory system, there are few studies that relate far-field responses evoked by angular acceleration to the vestibular system.

In this study we performed experiments designed to examine whether interpeak latencies of the BAER in rats depended upon temperature. (Rats are one species that have been widely used in studies in space, and this experiment at 1 G provides baseline data on BAERs in rats.) This led to experiments designed to examine whether interpeak latencies of responses evoked by angular acceleration show a dependence on temperature.

(We have also continued studies on another neural system in the rat, the thermoregulatory system, as altered by hypergravic fields. However, in this abstract an emphasis will be placed on our most recent vestibular studies.)

Methods Long-Evans male rats (250-500 grams) were used in this study. All surgical and recording procedures were performed on animals anesthetized with sodium pentobarbital. Recording electrodes (five stainless steel screws) were implanted in the skull: A recording screw was placed low in the mastoid bone on each side. A vertex recording screw was placed midway between lambda and bregma, and two ground screws were placed over the frontal sinus rostral to bregma. To measure brain temperature a calibrated bead thermistor (VECO) was placed subdurally opposite the vertex screw. Wire leads were secured to each recording screw and the thermistor. A nut was stereotaxically positioned equidistant between the external auditory meatus, and this nut and implanted hardware were fixed in place with dental acrylic. The nut was used to attach a piezoelectric crystal for delivering auditory clicks, and it also provided a reference point for angular acceleration.

The neural activity evoked by each stimulus modality was analyzed by averaging responses to stimuli. The electroencephalographic signal was amplified and sampled immediately after each stimulus. Final waveforms were the average of 128 or 512 responses using a laboratory microcomputer (Sierra Data Systems). Brain temperature was recorded at the onset and at the completion of averaging each waveform. After recording responses to both stimulus modalities at approximately 37 degrees centigrade, the brain temperature was allowed to fall 3 degrees. Responses were then recorded at the lower brain temperature.

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For recording responses to angular acceleration the head position of the anesthetized rat was maintained over the axis of platform rotation. This was accomplished by securing the nut (fixed in dental acrylic upon the animal) to a head holder, assuring consistent and repeatable placement of each animal with reference to the auditory meatus. Brief, rapid rotatory movements of the platform were used.

BAERs were recorded in response to bone-conducted stimuli using a piezoelectric crystal secured to the nut. Voltage pulses applied to the crystal induced clics that were conducted to the skull and stimulated the auditory system (Jones and Horowitz, J. Neurosci. Meth. 7:261-267, 1983).

Results Responses in six rats to auditory and vestibular stimuli were compared at two temperatures. It was observed that the peaks in the BAER recorded at a brain temperature of approximately 34 degrees centigrade occur later in time than the peaks from the 37 degree waveforms. In addition, the later peaks of the BAER recorded at the low temperature were more delayed than the earlier peaks, indicating that the interpeak latency increases as brain temperature is decreased.

Responses to angular accleration also show that later peaks in the evoked waveforms are associated with increases in interpeak latencies with decreasing temperature.

Discussion This study demonstrates that peak latencies of the BAER recorded from rats are sensitive to temperature changes, as has been previously shown in other species.

In addition, in this study we recorded increases interpeak latency of responses to brief angular accelerations when the temperature was lowered. However, additional experiments are necessary before we can with certainty attribute these responses to the vestibular system, and not the auditory or somatosensory systems. Preliminary experiments involving masking with white noise appear to exclude the auditory system as the generator of these responses. Preliminary experiments controlling for possible somatosensory input were also performed. No appreciable response was observed when the animal's head was fixed in place and the animal's body moved with the platform. Therefore, we tentatively conclude that these responses may be attributed to the vestibular system, and may be denoted brainstem vestibular evoked responses (BVERS). We plan to complete control experiments to determine if these responses can be attributed to the vestibular system with greater certainty.

(We have also completed a series of experiments on the thermoregulatory system of rats in a hypergravic field. Data was interpreted as indicating that there is a set-point shift of thermoregulatory controllers in the rat subjected to hypergravic fields. This transient shift in set-point was observed by altering the ambient temperature and monitoring core temperature of the rat in a hypergravic field.)

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NEURAL MECHANISMS BY WHICH GRAVITATIONAL STIMULI AND STRESS AFFECT THE SECRETION OF RENIN AND OTHER HORMONES

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There is evidence that serotonergic neurons in the dorsal raphe nucleus of the midbrain trigger increases in renin secretion by a pathway which passes from the nucleus to the ventral part of the hypothalamus. Our research has been directed at detecting how the signal gets from the hypothalamus to the kidneys to increase renin secretion, and at exploring the possibility that the serotonergic pathway, and more generally, the hypothalamus, are important in mediating the alterations in plasma renin activity produced by gravitationally and space flight-related stimuli such as tilting, alterations in extracellular fluid volume, and psychological stress.

A pharmacologic study was first conducted to determine whether the pathway from the hypothalamus to the kidneys was sympathetic. In rats, the increase in plasma renin activity produced by the serotonin-releasing drug p-chloroamphetamine (PCA) was found to be blocked when β-adrenergic receptors on the renin-secreting juxtaglomerular cells were blocked by L-propranolol, but not when the relatively inactive D-isomer of propranolol was injected as a control. The effect of chlorisondamine, a drug that blocks conduction in sympathetic ganglia, was also investigated. This drug increased plasma renin activity by itself, in all probability because it lowered blood pressure. However, PCA given to rats treated with chlorisondamine failed to produce any further increase in plasma renin activity. These data indicate that the pathway from the hypothalamus to the kidneys is indeed sympathetic.

We have also started to determine which hypothalamic nuclei mediate the response to PCA. The hypothalamic lesions that blocked the renin response were large in our previous experiments, and we sought to determine which regions within the large area destroyed by the lesions were specifically responsible for the effects on renin secretion. In one preliminary experiment, bilateral lesions of the paraventricular nuclei blocked the response, but so did lesions of the ventromedial nuclei. A repeat of this experiment with larger numbers of animals and a sham-operated control group is being carried out.

We have also conducted a series of preliminary experiments to be sure we have a readily reproducible psychological stimulus to renin secretion that can be used in rats. Previous experiments by Bahnson and Ganong indicated that exposure to a novel environment increased renin secretion. However, we found this response to be distressingly variable from one group of rats to another. We then explored the effect of immobilization by the relatively benign procedure of placing rats in clear plastic tubes. This stimulus regularly produced an increase in plasma renin activity in 10 minutes. Dorsal raphe lesions failed to alter the renin response to this kind of immobilization. However, it will be interesting to test the effect of paraventricular lesions on the response to immobilization.

The effects of various anesthetics on plasma renin activity were also explored in preparation for the tilting experiments. Equithesin and urethane increased plasma renin activity, but inactin produced little or no increase. Experiments testing the effects of 60° head-up tilt on plasma renin activity in inactin-anesthetized rats have been carried out, but the measurements have not yet been completed.

Finally, the relation of vasopressin-secreting neurons in the brain stem to the PCA response has been explored. Vasopressin-secreting neurons project from the paraventricular nuclei to the medulla and spinal cord, and are probably involved in cardiovascular control. The paraventricular nuclei were in the area destroyed by the large lesions in our previous studies. Therefore, we tested the effects of PCA on plasma renin activity in Brattleboro rats that are congenitally unable to produce vasopressin in their hypothalami. Brattleboro rats had larger rather than smaller responses to PCA. The cause of this supernormal response is being explored. Brattleboro rats have chronically elevated plasma renin activity at rest, and it may be that their renin-secreting cells become hyper-responsive due to chronic stimulation, like other chronically stimulated endocrine glands. We plan to investigate this possibility by testing renin secretion in Brattleboro rats in response to the β-adrenergic agonist isoproterenol, which acts directly on the juxtaglomerular cells, thus testing juxtaglomerular cell responsiveness and reserve. The *in vivo* responses will be supplemented by *in vitro* studies in which kidney slices from Brattleboro rats will be studied.

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NINTH ANNUAL NASA SPACE BIOLOGY SYMPOSIUM
Harpers Ferry, West Virginia, 7-9 November 1984

GRAVITY, BODY MASS AND COMPOSITION, AND METABOLIC RATE

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NASA Grant NSG-7336

A major objective of our project has been the systematic definition of metabolic rate and body composition as a function of sex and age in 5 species of common laboratory mammals, the mouse, hamster, rat, guinea pig and rabbit. The 100-fold body mass range represented is suitable for examination of scaling relationships.

To accomplish the objective, we have measured oxygen consumption and carbon dioxide production rates individually in 6 male and 6 female animals for each of 8 age cohorts ranging from 1 month to 2 years, and for each of the species. The animals were killed immediately after the respiratory gas exchange measurements, and dissected for determination of organ masses. The visceral organs of each animal were combined for water and fat content measurement by freeze-drying and petroleum ether extraction, respectively. Water and fat content of the skin and of the skinned, eviscerated carcass were similarly determined, and all of the dried, defatted body tissues from each animal were combined and comminuted to a homogeneous powder for elemental analyses.

The dried, defatted, whole-body powders were also analyzed for creatine content to measure body creatine mass. Inasmuch as about 97% of the body creatine is contained by the skeletal muscle cells of the body, in principle the body skeletal muscle mass may be computed from the body creatine mass if the intracellular skeletal muscle creatine concentration is known. However, intracellular creatine concentration varies as a function both of species and of age. Therefore, we undertook to measure creatine content of fat-free skeletal muscle samples from animals of various ages for our 5 species to provide appropriate values for computation of body skeletal muscle mass from body creatine mass by means of the relationship

$$\text{Fat-Free Skeletal Muscle Mass, g} = \frac{\text{Body Creatine Mass, g} \times 97}{\text{Creatine, g/100 g Fat-Free Muscle}}$$

The body skeletal muscle mass was expressed as a percentage of the fat-free body mass for each animal to provide a measure of muscularity for comparison among the various groups of different species, sex and age by 3-way analysis of variance. There was little to no sex difference in muscularity within the 5 species; however, there was a pronounced species difference. The mouse emerged as the most muscular, the rat, rabbit and hamster exhibited intermediate muscularity, and the guinea pig was the least muscular of the 5 species.

There was also a marked difference in muscularity as a function of age within each species. It tended to be low in the young animals 1 month of age,

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reached a peak at 2-3 months, and then declined in exponential fashion out to 2 years of age. The attainment of maximal muscularity at 2-3 months of age agrees well with the onset of puberty, in contrast to the attainment of metabolic maturity at age 6-8 months or the attainment of mature body mass at about 1 year of age.

Several general conclusions may be drawn from the results. It is evident that among these small mammals there is no indication of scaling of muscularity to body size, despite the 100-fold difference in body mass represented. Thus, it appears that in this size range of mammals mechanical loading by Earth gravity has not been as important a natural selection factor for muscularity as other requirements on the animal. For example, the correspondence noted between muscular maturity and sexual maturity may well indicate a primary value of optimal mobility for species survival.

The observation that the proportion of the fat-free body mass represented by the skeletal musculature seems to reach a pronounced peak value at age 2-3 months and then declines, carries the implication that the fraction of the fat-free body represented by other body components in older animals must increase complementarily. We suggest that in all likelihood it may be the supporting components of the body, the connective tissue and skeleton, which are proportionately greater as the animals continue to grow. Future analysis of additional body composition data from our 5-species series is expected to provide a test for this hypothesis.

Finally, we may conclude that under normal gravity conditions muscularity in small laboratory mammals displays large, systematic variation as a function both of species and age. Therefore, it is important to take this variation into account when such animals are subjects of experiments to study the effects of altered gravitational loading on the skeletal musculature of the mammal.

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HOMEOSTASIS IN PRIMATES IN THE HYPERDYNAMIC ENVIRONMENT

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NASA Grant: NAGW-309

Using the small diurnal squirrel monkey (Saimiri sciureus) as a non-human primate model, this research program has been investigating the influence of altered acceleration environments upon various homeostatic physiological and behavioral systems. Such systems have included food and water intake, temperature regulation, circadian rhythms and sleep state. During the last year, this research program has focused upon two areas. First, we have examined the influence of chronic centrifugation upon the homeostatic regulation of the circadian timekeeping system. Second, in a countinuing series of experiments focused upon the interactions of body temperature regulation and the behavioral state of arousal; we have performed a preliminary evaluation of the influence of cephalic fluid shifts induced by lower body positive air pressure (LBPP), upon these systems.

Few environmental parameters are capable of modifying the homeostatic regulation of the mammalian circadian timekeeping system. This first study was designed to examine the possible effects of hyperdynamic environment (+G) on the circadian timekeeping system of the squirrel monkey. Eight unrestrained adult male monkeys ranging from 900 to 1200 gm, were exposed to 1 G for 21 days, followed by 1.5 G for 14 days, 2.0 G for 33 days and 1 G again for 21 days. During the two 1 G phases and the 2.0 G phase, the animals were exposed to constant light (LL) for 8-14 days, in addition to the 24-hour light-dark cycle (LD 12: 12) present during the remainder of the experiment. All animals were exposed to +G simultaneously on an 18 ft diameter centrifuge. The animals were allowed food and water ad lib, which was monitored electronically every 30 minutes. The values were stored on a microcomputer for later analysis.

In all three acceleration fields, the animals demonstrated prominent circadian rhythms in both feeding and drinking. In LD, both rhythms were always entrained to the 24-hour light cycle. However, a phase-angle shift occurred between the physiological rhythms and the LD synchronizer when the animals were in the hyperdynamic environment. This phase delay suggests a possible increase in the period of the circadian pacemakers as a result of the +G. When the animals were exposed to LL while they were at 2.0 G, the rhythms not only persisted with a free-running period different from 24-hours, the mean free-running period of all 8 animals was longer ($p<0.05$) than when the animals were at 1 G both before and after the hyperdynamic exposure. This study demonstrated that the circadian timekeeping system of these primates is functional in the hyperdynamic environment, however, some of its components appear to be regulated at different homeostatic levels.

The second series of experiments have utilized cephalic fluid shifts, induced by LBPP, which are known to influence various physiological systems (i.e., cardiovascular and renal). In earlier experiments, we observed an apparent change in the arousal state of primates in such LBPP conditions. This study was designed to examine the effects of LBPP on arousal state and body temperature level, which is normally correlated with sleep. Eight male squirrel monkeys weighing 950 to 1100 gm were exposed to 40 mm Hg LBPP for 90 minutes between the day time hours of 13:00 and 15:00. Each monkey was placed in a specially modified restraint chair to which they were highly trained. This system enclosed the lower portion of the animal's body. A customized neoprene rubber belt worn above the hips sealed a partition isolating the lower half of the body. Each animal was given several hours to accommodate to the isolated recording environment prior to data collection. Monkeys in the same apparatus, except not wearing the rubber belt, and subjected to equivalent air flow, but no pressure, served as controls. Both air temperature and pressure supplied to the LBPP apparatus were regulated and constantly monitored. Sleep parameters were polygraphically recorded from 4 animals chronically implanted for sleep recording. In all animals, a video camera was used to observe the animals apparent state of arousal. LBPP resulted in an approximate 0.7°C decrease in DBT ($p < 0.01$). However, although on video some animals appeared drowsy during LBPP, sleep recording revealed no significant changes in state of arousal. Thus, the physiological mechanisms underlying this lowering of body temperature can be independent of the arousal state.

Our current efforts are aimed at continuing and extending these studies on integrative and regulatory physiology in altered ambient acceleration fields. Future experiments will further define these systems responses, their mechanisms of perception and action and the consequence of such responses on the organisms adaptability to such environments.

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A DEVICE FOR APPLYING CONTROLLED FORCES TO TISSUES GROWING IN STERIAL CULTURE

Philip Lintilhac
University of Vermont
Burlington, Vermont

Abstract not distributed at the meeting.

C. MECHANISMS OF PLANT RESPONSES TO GRAVITY

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GRAVITROPISM IN LEAFY DICOT STEMS

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NASA Grant NSG-7567

Our research during the past year includes the projects of two doctoral students plus a small project of the principal investigator. A research technician, Linda Gillespie, assists with all three projects.

Possible Changes in Orientation of Cellulose Microfibrils in Cell Walls of Gravistimulated Dicot Stems (Rosemary White). The problem of microfibril orientation is intriguing based on our initial observations several years ago that stems restrained to a horizontal position for two or three days and then suddenly released form a sharp bend, and that, during this bending, cells on the top of such a stem become significantly shorter while those on the bottom become longer. This sudden change in cell dimensions could easily be determined by orientation of cellulose microfibrils in the walls.

Much of our time on this project during the past year has been spent in tooling up for the study. A polarizing research microscope with rotating stage and associated camera equipment has finally been ordered, and techniques of fixation and preparation of specimens have been perfected. Results are just beginning to come in as this report is being written (August 20, 1984). White presented a poster at the recent Plant Physiology Meetings in Davis, California, and she received several excellent suggestions and even offers of help and cooperation from people who saw the poster. Malcolm Brown at the University of Texas at Austin has already obtained preliminary results on microfibril orientations during gravitropic bending (different system from ours). He and White have discussed possible cooperative efforts.

White has also extended her work with ethephon reported last year. Her most important finding was that the ethephon effects were apparently nothing more than acid growth. Acid ethephon solutions or acid without ethephon caused elongation of stem tissues where they were applied; stems bent away from the side of application. A most interesting finding, however, was that acid solutions applied to the bottom of horizontal stems greatly delayed bending. White has repeated these experiments several times to satisfy herself that the results are real. The work is now ready to be written up for publication.

We are discussing the possibility of coorperating with A. Carl Leopold in the investigation of electric field changes around plant organs during gravistimulation. This work has been delayed while the necessary equipment is being developed at the National Facility for the Vibrating Probes at Woods Hole, but we hope to carry out some preliminary experiments with modified plant material and equipment in the near future.

Studies of tissue sensitivity changes during gravitropic bending (Patricia Rorabaugh). Nearly a hundred experiments have been completed

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using a system in which horizontal soybean hypocotyls (with and without cotyledons) are allowed to respond to gravity while immersed in buffered solutions of auxin, calcium chloride, or possibly other substances in future research. The two sides of the hypocotyl exhibit a different initial sensitivity to gravitropic stimulation and to auxin solutions. When the cotyledons of 4-day-old horizontal soybean hypocotyls are hanging down, response to gravity is twice that of when the cotyledons are in an upright position. IAA concentrations of about 10^{-7} to 10^{-4} M inhibit gravitropic bending. Vertical controls are also used in these studies. When the horizontal hypocotyls are scrubbed on top to remove the cuticle, much lower concentrations of IAA (at least as low as 10^{-8} and possibly 10^{-10} M) inhibit upward bending. At the highest auxin concentrations, hypocotyls scrubbed on top bend downward. Apparently, auxin moving into the upper tissues prevents them from stopping their growth (to use an unavoidable double negative). When hypocotyls are scrubbed on the bottom, there is virtually no response to auxin; upward bending occurs at about the same rate at all concentrations, although bending is reduced at the highest auxin levels. Total growth (measured along a centered line of the hypocotyl) was observed in all of these experiments and found to be virtually uninfluenced by any auxin concentration. (Scrubbing stimulates growth in all experiments.) When vertical hypocotyls were pretreated with 10^{-8} m IAA for relatively short time intervals (e.g. 30 min), there was a significantly increased positive gravitropic response to the same concentration of auxin when the hypocotyls were turned to the horizontal. So far, this may be our best evidence for an effect of auxin on sensitivity of the tissue. We hope to pursue it further. Ca^{2+} inhibits both bending and elongation as concentrations are increased above a threshold level (about 10^{-3} M).

A striking observation from these experiments is the fantastic range of auxin concentrations over which effects are observed: from perhaps 10^{-8} to 10^{-4} M IAA. The Cholodny-Went theory suggests that gravitropic bending occurs in response to a gradient in auxin concentration across a gravitroping stem. Yet endogenous gradients have been either impossible to observe (in certain studies) or only very small (perhaps 40% auxin on top, 60% on the bottom). It will be interesting to see how this can be reconciled with our observations of responses over nearly four orders of magnitude of auxin concentration. To study this, it will be essential to measure the amount of auxin that gets into the tissue on the top and on the bottom of a horizontal hypocotyl.

The Mechanics of Gravitropic Bending in Dicot Stems (Frank B. Salisbury). Some studies with castor bean stems, initiated while the principal investigator was on sabbatical leave in Israel and Austria, were reported last year. These studies have been continued in Utah, but at this time there is little additional information to report. Only a few repetitions of experiments done in Austria have been completed. Evidence is developing for different water potentials in the pith tissues on top and bottom of a gravitroping stem. These tissues exert a pressure in the stem against the tension of outer cortical and vascular tissues, and so these tissues may well be controlling normal growth as well as gravitropic bending. Techniques for studying these matters are continuing to develop, and it is hoped that there will be more to report next year.

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MECHANICAL STRESS REGULATION OF PLANT GROWTH AND DEVELOPMENT

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NASA Grant: NSG 7278

Periodic seismic (shaking) stresses inhibit growth in height, fresh weight, dry weight, and leaf area of vegetative soybean as well as many other species of higher plants. It is distinctly possible that the mechanical vibrations normally attending spacecraft operation, astronaut activity, etc., not only will alter plant growth and development, but will mimic pulses of gravity that would confound biological experiments in microgravity. One growth response of soybean to seismic stress in a controlled environment is a temporary decline in net photosynthetic productivity, as indicated by a 22% reduction in plant dry weight and a 17% decrease in leaf area relative to undisturbed controls after 15 days of 3-times-daily gyratory shaking for 5 minutes at 240 rpm. Growth dynamics analysis was used to determine to what extent the seismic stress-induced reduction in photosynthetic productivity was due to less photosynthetic surface, and to what extent to lower efficiency of assimulation. All of the reduction in relative growth rate (RGR) caused by periodic seismic treatment was due to inhibition of net assimilation rate (NAR) and not to leaf area ratio (LAR), which are related according to RGR=NAR x LAR.

Seismic stress reduced shoot transpiration rate 17% and 15% during the first and second 45 minute periods following a given treatment. Shaken plants also had a 36% greater leaf water potential 30 minutes after treatment. Continuous measurement of whole plant photosynthetic rate showed that a decline in CO_2 fixation began within seconds after the onset of shaking treatment and continued to decline to 16% less than that of controls 20 minutes after shaking, after which gradual recovery of photosynthesis began. Photosynthetic assimilation recovered completely before the next treatment 5 hours later. The transitory decrease in photosynthetic rate was due entirely to a two-fold increase in stomatal resistance to CO_2 by the abaxial leaf surface. Mesophyll resistance was not significantly affected by periodic seismic treatment. Thus, temporary stomatal aperture reduction and decreased CO_2 fixation were responsible for the lower dry weight of seismic-stressed plants growing in a controlled environment.

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MUTANT PEAS AS PROBES IN THE UNDERSTANDING OF GROWTH AND GRAVITROPISM

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We are currently using 2 mutants of Pism sativum as aids in the understanding of growth patterns and gravitropism. The first mutant, "CREEP" grows normally up to the first internode stage, and then begins to grow plagiotropically. The upper internodes bend slowly downward according to a programmed sequence which follows circumnutation of the previous internode and opening of the previous leaves, but preceeds expansion of the previous leaves. The bending is partially inhibited by excission of the opposing stipules, a fact which suggests that some messenger may normally come from the stipules which permits the plagiotropic habit. Experiments are planned to see if the stipules have any effect on normal negative orthogravitropism in wild type plants.

The second mutant, "AGEOTROPUM", is gravitropically incompetant when grown etiolated, in the dark. If the etiolated plants are illuminated with white light, the stems become gravitropically competant, but the roots do not. If the plants are grown in the light in particulate medium, some of the secondary roots, growing randomly, emerge into the air, and, turn and grow downward. Previous workers have suggested that this is because they become illuminated and thus are converted to gravitropically competent organs. We have found that they still remain ageotropic, but that they grow away from the drier air toward the moist soil. They thus may be said to be positively hydrotropic.

When etiolated AGEOTROPUM plants are illuminated, the shoots then become able to respond to gravity in a normal, negatively orthogravitropic manner. We show that the response is to red light and is reversed by far red light. This sequential photoreversibility demonstrates that phytochrome is the pertinent light absorbing pigment. If the stems are made gravi-competent with red light, the red light effect also can be reversed by treatment of the plants with the calmodulin inhibitor, chlorpromazine (1 uM). This suggests that one site of the mutation may involve calcium metabolism. We have looked for redistribution of the intra-organ mobile calcium, and found that whereas normal upward redistribution occurs in wild type and red light induced AGEOTROPUM, it does not occur in the dark grown non-induced mutant. In analysing these data, we conclude that the mutation may involve one or more of the following: 1) release of sequestered calcium for redistribution; 2)

radial transport of released calcium; or 3) net calcium flux in the upward direction. Experiments are currently planned to differentiate between these possibilities.

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PLANT GROWTH BIOPHYSICS: THE BASIS FOR GROWTH ASYMMETRY INDUCED BY GRAVITY

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NASA Grant NAGW-480

Plant growth requires water absorption and irreversible wall expansion. When gravity induces a plant stem to grow upwards, its effect on growth must be mediated via asymmetrical alteration of one or more of the cell parameters that control water transport or irreversible wall expansion. The goal of this project is to identify and quantify the physical properties altered by gravity when plant stems grow upwards.

The first step in this research has been to characterize the spacial and temporal details of the growth response to gravity. For this work, dark-grown stems of young cucumber (Cucumis sativus L. cv Burpee's Pickler) seedlings were marked with black ink at 2-mm intervals throughout the length of the growing region. Growth of the stem in vertical and horizontal positions was recorded at 15-min intervals by time-lapse photography. The photographs were projected onto a digitizing tablet interfaced to a microcomputer, and the x/y positions of the marks on the lower and upper surfaces of the stem were recorded by the computer. A program that uses a cubic spline fitting algorithm was used to calculate the growth rate and curvature of the various regions of the stem as a function of time after horizontal placement. The responses of 20 plants were averaged to reduce the effect of random noise and variability. The results show that the cucumber stems begin curving in less than 15 min and within 45 min develop a 70-85° curvature. Both the upper and lower sides of the stem react. The maximum asymmetry in growth develops at 30 min after stimulation, when the upper side ceases growth almost entirely whereas the lower side doubles its growth rate (compared to horizontal growth rates). These disparate growth responses on the upper and lower sides occur simultaneously along the entire length of the growing region. The initial growth response does not migrate from the apex towards the base of the stem.

One component of the driving force for growth is the osmotic pressure of the cell contents. To test whether cell osmotic pressure was altered by gravity, cucumber stems were bisected during the period of maximal gravitropic response. There was no difference in the osmotic pressure of cell sap expressed from the two halves. Similar results were obtained when only the epidermis was used to obtain expressed cell sap. Thus the hypothesis that gravitropism involves an asymmetry in bulk osmotic pressure can be ruled out.

A new technique for measuring the yielding properties of the cell wall has been developed and tested using growing pea stems. The method involves isolating a growing tissue from an external water supply. Continued wall loosening then induces stress relaxation in the cell walls and a concomitant reduction in the turgor pressure. Analysis shows that the rate of stress relaxation is controlled predominantly by the apparent wall extensibility and the volumetric elastic modulus of the cell. The final turgor pressure attained after completion of stress relaxation is the yield threshold.

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An Indirect Role for Ethylene in Shoot-Inversion Release of Apical Dominance in *Pharbitis nil*

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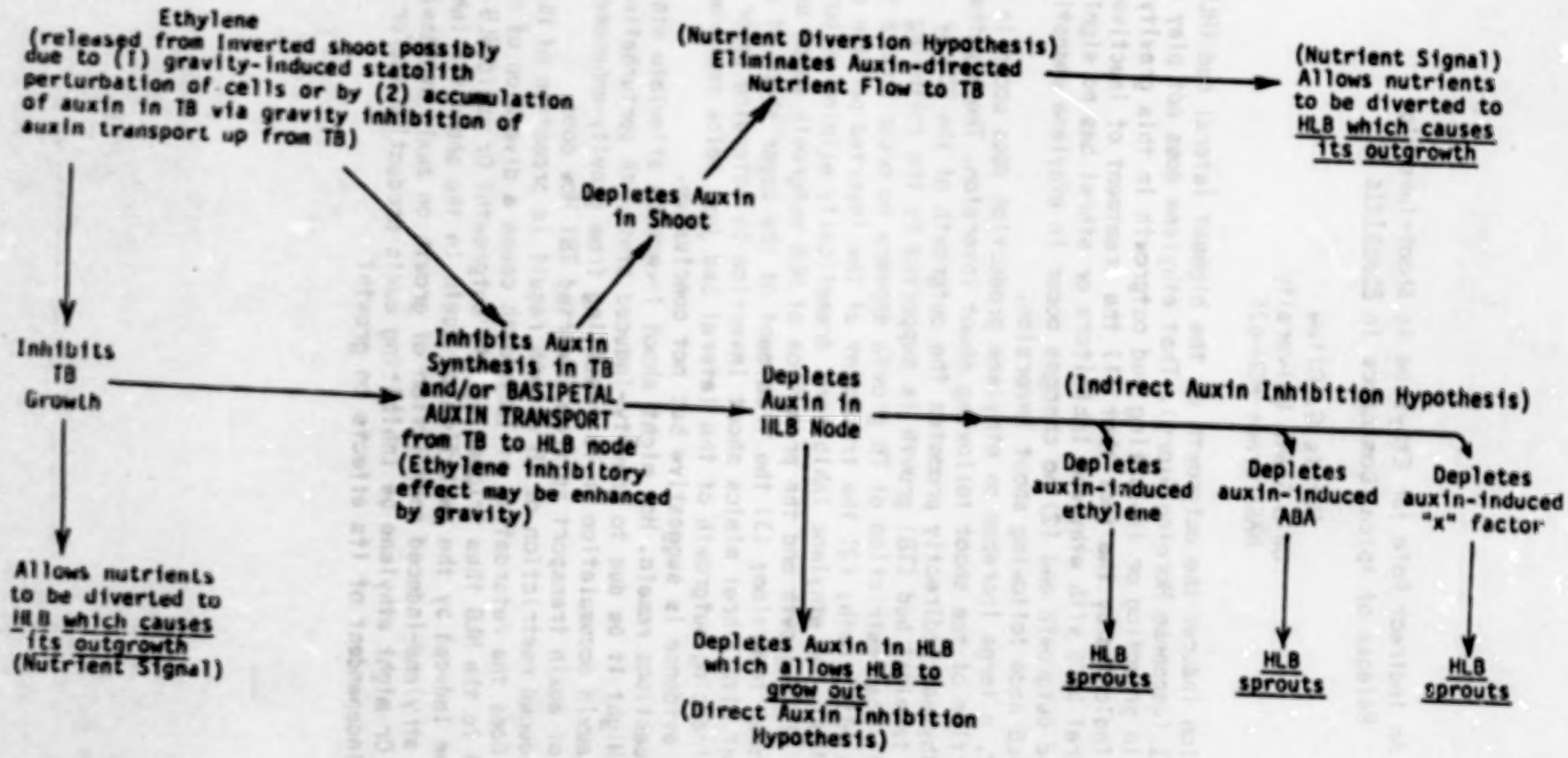
NASA Grant NSG-625

Shoot inversion induces the outgrowth of the highest lateral bud (HLB) in *Pharbitis nil* (Japanese Morning Glory). That ethylene does not play a direct role in promoting or inhibiting bud outgrowth in this gravity response is indicated by the fact that (1) the treatment of inactive or induced lateral buds with ethylene inhibitors or ethrel has no significant effect on bud outgrowth and (2) no changes occur in ethylene emanation in the HLB or HLB node following shoot inversion.

However, a large increase on ethylene production does occur in the inverted portion of the shoot following shoot inversion. The hypothesis that this ethylene indirectly promotes the outgrowth of the HLB by restricting terminal bud (TB) growth is supported by the following evidence: (1) the restriction of TB growth appears to occur before the beginning of HLB growth; (2) the treatment of the inverted portion of the shoot with $AgNO_3$, an ethylene inhibitor, dramatically eliminates both the restriction of TB growth and the promotion of HLB outgrowth which usually accompany shoot inversion; (3) the treatment of the upper shoot of an upright plant with ethrel mimics shoot inversion by retarding upper shoot growth and inducing outgrowth of the lateral bud just below the treated region. The evidence is suggestive but not conclusive.

Many questions remain. How might shoot inversion stimulate ethylene synthesis? Might it be due to gravity-induced statolith perturbation of the cell or to auxin accumulation in TB resulting from gravity-enhanced inhibition of auxin transport up from inverted TB? How does ethylene-induced restriction of TB growth result in promotion of HLB outgrowth? Does the retardation of TB growth cause a diversion of nutrients from the TB to the HLB thus triggering its outgrowth? Or could HLB outgrowth be induced by the depletion of auxin in the shoot via inhibitory effects of ethylene-induced restriction of growth on auxin synthesis and/or transport? Or might ethylene be inhibiting auxin production and/or transport independent of its effects on growth?

POSSIBLE MECHANISMS BY WHICH ETHYLENE RELEASED BY SHOOT INVERSION MAY INDIRECTLY PROMOTE OUTGROWTH OF THE HIGHEST LATERAL BUD (HLB)


Ethylene
(released from inverted shoot possibly due to (1) gravity-induced statolith perturbation of cells or by (2) accumulation of auxin in TB via gravity inhibition of auxin transport up from TB)
(Nutrient Diversion Hypothesis)
Eliminates Auxin-directed Nutrient Flow to TB
(Nutrient Signal)
Allows nutrients to be diverted to HLB which causes its outgrowth
Depletes Auxin in Shoot
Inhibits TB Growth
Inhibits Auxin Synthesis in TB and/or BASIPETAL AUXIN TRANSPORT from TB to HLB node (Ethylene inhibitory effect may be enhanced by gravity)
Depletes Auxin in HLB Node
(Indirect Auxin Inhibition Hypothesis)
Depletes auxin-induced ethylene
Depletes auxin-induced ABA
Depletes auxin-induced "x" factor
HLB sprouts
HLB sprouts
HLB sprouts
Allows nutrients to be diverted to HLB which causes its outgrowth
(Nutrient Signal)
Depletes Auxin in HLB which allows HLB to grow out
(Direct Auxin Inhibition Hypothesis)

TB = terminal bud

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GRAVITY PERCEPTION AND RESPONSE IN SHOOTS OF CEREAL GRASSES

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NASA Grant NAGW-34

Our NASA Space Biology research this past year has been focussed on two primary components of the gravitropic curvature response in cereal grass pulvini: (1) gravity perception and (2) mechanism of response following the transduction phase. A summary of our results is as follows:

(1) GRAVITY PERCEPTION

With a more sensitive recorder (Gould Instruments, Model 110) and a precisely counterpoised angular recording transducer arm, we have shown that upward bending in mesocotyls of maize seedlings (Zea mays) begins, on average, within 3 minutes. This is within the time that full asymmetry in free IAA is established (Bob Bandurski, personal communication).

In oat (Avena sativa) and barley (Hordeum vulgare) shoots, upward bending in leaf-sheath pulvini at next-to-last nodes begins, also, on average within 3 minutes, when measurements are made at 12 midnight for barley; and within 20 minutes for oats when measurements are made at 8 A.M. These are minimum lag-time values. In contrast, the time for initiation of upward bending may be as long as 100 minutes for barley when measurements are made at 12 noon; and 160 minutes for oats when measurements are made at 12 midnight. These are maximum lag-time values. It turns out that there is a Circadian rhythm in this response in both barley and oat shoots obtained from plants grown at 360 μmol m^{-2} s^{-1} light intensity, 16 h light/8 h dark, and 21°C. in a plant growth chamber. The barley and oat shoots are out of phase by ca. 12 h in their max./min. lag phase times. Our results indicate that it is very important for investigators to make such measurements at the same times of day when comparative experiments are conducted! The reason for the differences in lag times that we have measured is not known, but it may be related to changes in levels of photosynthate (sugars) that occur during the light and dark periods of a 24-h cycle. We have shown previously, that sucrose is an important substrate for the gravitropic upward bending response in leaf-sheath pulvini of isolated shoots of barley and oats.

(2) MECHANISM OF RESPONSE

In light of the fact that protein synthesis is necessary for a negative gravitropic curvature response to occur in grass leaf-sheath pulvini, some proteins from gravistimulated leaf-sheath pulvini of barley were analyzed by SDS-PAGE and 2-d gel electrophoresis. The SDS-PAGE band patterns indicate that at least five proteins were increased in tissues derived from bottom halves of the gravistimulated pulvini (Mol. Wt. ranges of 32, 39, 57, 105 & 110 Kd) and one at 81 Kd increased sharply in the upper halves. It appears that some of these proteins may be wall-loosening enzymes which are involved in the differential cell elongation response that occurs in gravistimulated pulvini.

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Because we have shown that SDS-PAGE protein banding patterns are changed in cereal grass leaf-sheath pulvini extracts as a result of gravistimulation, we have analyzed protein extracts of upper and lower halves of oat pulvini from prostrate plants, measuring total quantity of protein extracted as well as the activities of four enzymes: endocellulase, endoarabinoxylanase, β-glucosidase, and invertase. β-glucosidase activity decreased steadily during the time period studies (0,3,6,12, and 24 h of gravistimulation). Arabinoxylanase activity in buffer-soluble fractions showed no striking changes during the time-course of the experiment. In contrast, buffer-soluble invertase activity increased markedly, and within 3 hrs., in the lower halves of the graviresponding pulvini. Further, cellulase activity, for both salt-soluble and buffer-soluble cellulase, increased markedly in the upper halves of the graviresponding pulvini. What does all this mean or suggest? The enhanced cellulase activities in the upper halves may open up sites for new wall material synthesis within existing cellulose molecules. The resulting strengthening of cell walls in the upper half of the pulvinus could reduce the tendency for this region of the stem to break under the torsion required to lift a prostrate oat plant to a vertical position. The enhanced invertase activity in the lower halves could provide needed hexose (D-glucose and D-fructose) from photosynthate (sucrose) made in the chlorophyll-containing starch statoliths in the pulvinus for cell wall biosynthesis that is required for upward bending to occur in this system.

SESSION II. ROLE OF GRAVITY IN DEVELOPMENT

B. ANIMAL GROWTH AND DEVELOPMENT

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Simulated Microgravity as a Probe For Understanding the Mechanisms of Early Pattern Specification

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NAGW-60

Early pattern specification (e.g., axial structure morphogenesis, dorsal ventral polarity, etc.) was monitored in amphibian eggs which were subjected to microgravity simulation by constant rotation on a horizontal clinostat.

In contrast to previous clinostat experiments, rotation was initiated either prior to fertilization or immediately thereafter. Large proportions of clinostated eggs developed normal axial structures. The mechanisms which specify axial structure morphogenesis probably do not involve gravity-driven rearrangements of the egg cytoplasm. A model which employs a multiple set of signals for specifying early pattern will be discussed.

Effects of microgravity simulation on the earliest post-fertilization pattern specification event—dorsal/ventral polarization—was analyzed in detail. The natural relationship of the dorsal polarity, vis-a-vis the site of sperm entry, was uncoupled in clinostated eggs. That observation was interpreted to indicate that one of the current models of the mechanism of egg polarization is probably inappropriate. Other models were therefore developed and they will be discussed. As a general mechanism for explaining the manner in which regional developmental patterns emerge from the initial, radially symmetrial egg, the "density compartment model" will be described. The identification of the various zones or compartments of egg cytoplasm using inverted eggs will be explained.

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DEVELOPMENT and MATURATION of the NEUROMUSCULAR JUNCTION IN CELL CULTURE UNDER CONDITIONS OF SIMULATED ZERO-GRAVITY

I. Effects of Clinostat Rotation on Morphology of Nerve and Muscle Cells

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NASA Grant NAGW-539

Introduction & Objectives

As space-flight durations are extended and plans for space colonization progress from planning to implementation, understanding the impact of prolonged exposure of biosystems to hypogravity and zero-gravitational (0g) forces becomes essential to their survival and development under these novel conditions. It is already well-documented that prolonged exposure of humans to 0g conditions during space flights results in muscle weakness and atrophy (4,2,11). Similarly, it is known that biosystems, from plants to vertebrate animals, sense gravitational signals which influence their development and maturation (6-9). It is therefore logical to predict that development and maturation, under 0g forces, may be significantly affected. The objective of this project is to examine whether alterations in gravitational conditions alter the normal development and interactions of nerve and muscle cells grown in culture.

The first phase of this study is concerned with defining clinostat conditions, to simulate 0g, which produce changes in cell morphology and growth patterns. Subsequent phases will utilize these conditions for the study of: (a) specific molecules involved in the differentiation and function of nerve and muscle cells, (b) changes in synaptic transmission, and (c) properties of single ion channels, responsible for signal transduction, in this in-vitro neuromuscular unit.

Materials & Methods

Cell cultures: Nerve and muscle cells are obtained from Xenopus laevis embryos according to previously published methods (3). Briefly, myocytes and spinal neurons are isolated by mechanical and biochemical dissection under sterile conditions. Known cell densities are seeded into sealable culture chambers constructed from glass slides and rings sandwiched between glass coverslips and assembled with silicon grease. The chambers are sealed to exclude air bubbles and mounted on a clinostat for rotation. Culture chambers are removed, at specified times, for microscopic observations and video-taping. Control cultures consist of sister cultures which remain stationary under identical growth and ambient conditions.

Clinostat construction and rotational speeds: Clinostats were modified from designs of Tremor & Souza (10) and Wolgemuth (oral communication). The clinostat consists of a constant-speed motor with removable gearboxes (Japan Servo Co.) which is shock-mounted, to reduce vibrations, on a vise. A culture chamber carrier is mounted on the gear-box shaft so that culture chambers can be rotated with the floor of the chamber parallel or perpendicular to the axis of clinostat rotation. In addition, the shaft of the clinostat motor can be positioned to be either parallel or perpendicular to the vector of gravity. All clinostat rotations took place at room temperature and under virtual anaerobic conditions at which these cells develop normally in culture (1,5).

Observations & measurements: At specified intervals, ranging from 12 to 120 hrs, cultures were removed from the clinostat holders and examined under phase-contrast microscopy (Zeiss, IM35, 400 to 600x). Attention was focused on cell

size, shape, density of yolk platelets, presence of striations, size and shape of nucleus and its nucleoli, and occurrence and size of neurites. Cultures were remounted on the clinostat, within 30 minutes, to continue the rotation regime. Initial measurements were done on randomly selected cells from sister cultures. More extensive measurements will be carried out after the acquisition of an image analysis system.

Results & Interpretations

Effects of clinostat rotation on myocyte morphology: The developmental changes in myocyte morphology, seen in control stationary cultures, follow a stereotypic progression commencing with the adhesion of the myoball to the culture dish substratum and ending with the formation of polygonal shape, virtually devoid of yolk platelets; the presence of a well-defined nucleus usually containing two nucleoli; and a strong expression of contractile proteins evidenced by typical striation patterns.

In contrast, myocytes from sister cultures subjected to prolonged (48-120 hrs) rotations on a horizontal clinostat, at speeds varying from ca. 15 to 100 Hz, exhibit a spicule-like morphology characterized by a 5-10 fold assymetry in cell axes (compared to the nearly circular morphology of control cells); a relative sparseness in the presence of striations which are frequently replaced by "streaking" cytoplasm; the relative abundance of yolk platelets; and the presence of an enlarged nucleus containing multiple nucleoli. These changes were not observed when the axis of rotation of the clinostat was parallel to the vector of gravity (i.e. perpendicular to the horizontal plane). These findings were more common when in cultures in which the flat aspect of the cells was perpendicular to GV. These cells experienced rotation (a "tumbling" motion) through, rather than parallel to, the flat aspect of the cell. Characteristics of spinal neurons, grown in co-culture with myocytes, were not so distinct from controls. Under clinostat conditions which showed the most pronounced changes in myocytes, the following changes were observed in neurons: neurite diameter was larger, growth cones appeared to be flattened and to occupy a larger surface area, and neurite lengths were shorter than in control cultures.

In summary, preliminary data show that rotation of cocultures of nerve and muscle cells results in morphologic changes which are predicted to significantly alter the functional interactions between the elements of a prototypic synapse. It is further predicted that similar alterations may occur in central synapses which may therefore affect the development of the central nervous system when subjected to altered gravitational conditions. It is impossible, at present, to assess the fidelity with which clinostat rotation simulates zero- or hypo-gravity encountered in space. The differential effect of speed of rotation and cell orientation, as reported here and elsewhere for other biosystems, suggests a considerable sensitivity of cells to altered gravitational forces. Calibration of clinostat fidelity, in simulating such altered conditions, requires that biosystems be subjected to the gravitational forces which are actually encountered during space flights.

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Effects of Simulated Weightlessness on Mammalian Development
II. Meiotic Maturation of Mouse Oocytes During Clinostat Rotation

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NASA Grant NAGW-346

In order to understand the role of gravity in basic cellular processes that are important during development, we are examining the effects of a simulated microgravity environment on mammalian gametes and early embryos cultured in vitro. A microgravity environment is simulated by use of a clinostat, which essentially reorients cells relative to the gravity vector. Initial studies have focused on assessing the effects of clinostat rotation on the meiotic progression of mouse oocytes.

The clinostat used in these studies is based upon the design originally developed by Tremor and Souza (Space Life Sci. 3: 179-191, 1972) and has been modified throughout the course of this work. Initial modifications centered on providing the unique in vitro culture requirements of mammalian oocytes and embryos: 37°C temperature, constant humidity, and a 5% CO_2 in air environment. A second major technical consideration lay in the selection of culture dish which was of small diameter, non-toxic to the cells, and would permit gas exchange. Sterile 96-well Micro Test III tissue culture plates are currently being used. They are cut in half and re-sterilized under UV light. Culture media is placed in the center of the well and overlaid with paraffin oil. The paraffin oil allows an exchange of gases, provides a barrier against contamination, lowers the evaporation rate of the media, and prevents the media from draining out of the center well when the vessel is mounted on the clinostat. A recent modification of our original design permits simultaneous horizontal (experimental) and vertical (control) rotations. This was accomplished by installing two bevel gears set at a perpendicular angle to each other. Parallel cultures are thus exposed to virtually identical conditions except for the direction of rotation relative to the gravity vector.

Oocytes are recovered from ovarian follicles from young, sexually mature female Swiss Webster mice. They are cultured for 16 hours under static (control), horizontal axis of rotation (experimental), and vertical axis of rotation (control) conditions. The oocytes are removed from the culture system and observed immediately under the dissecting microscope for polar body formation and gross morphological appearance, including any obvious cellular abnormalities such as a granular or necrotic cytoplasm, rupture of the zona pellucida, fragmentation, clumping of surrounding follicle cells, etc. No consistent alterations in any of the above properties were observed at any of the rotation speeds or conditions used.

The oocytes are then processed for cytogenetic analysis, according to a modified procedure described by Mizoguchi and Dukelow (J. med. Primat. 10: 180-186). These preparations are then

scored for germinal vesicle breakdown and progression through meiosis to Metaphase I or Metaphase II. Cytogenetic evaluation can also be made, particularly with respect to chromosome number and obvious fragmentation or structural rearrangement of the chromosomes.

Rates of germinal vesicle breakdown were similar in all experimental and control conditions. That is, clinostat rotation did not interfere with the oocyte's capability to resume meiosis. This was not surprising in light of the current belief that the trigger for resumption of meiosis after release from the follicle acts within 30 minutes. In this situation, any effects of altering the cells' orientation relative to the gravity vector would have to have been elicited almost immediately.

The efficiency of Metaphase II formation was comparable among static (control) oocytes, vertically rotated (control) oocytes, and horizontally rotated (experimental) oocytes at ¼, 1, 10, and 30 RPM. In contrast, at 100 RPM with horizontal rotation (but not vertical rotation), a decrease in the number of oocytes reaching the Metaphase II stage after 16 hours was noted. This inhibition was significant by Chi-square analysis. It would be of interest to determine whether this inhibition is due to an irreversible block of a proportion of oocytes in the Metaphase I stage or to a lag in the timing of the progression of meiotic disjunctive events.

Since fertilization occurs with the oocyte in the Metaphase II stage, progression to this stage in a timely sequence is an absolute prerequisite for normal fertilization and subsequent disjunction of the chromosomes to ensue. Inhibition in either the chronology or efficiency of meiotic maturation would thus affect normal fertilization and reproductive potential.

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EFFECTS OF IN VIVO AND IN VITRO EXPOSURE TO EXCESS GRAVITY ON GROWTH AND DIFFERENTIATION OF MAMMALIAN EMBRYOS

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NASA Grant NAGW-438

Over the past few years, this laboratory has been carrying out studies on the development of embryonic mouse tissues exposed to excess gravity in vitro and in vivo. Previously, a significant suppression of morphogenesis was found in mouse limb buds of various gestational ages exposed to 2.6G in organ culture (Duke, 1983). A similar suppression was seen in limb buds cultured under 3G. In 13 day limbs exposed to 3G for two days, and then cultured at 1G for 2-4 days, suppression of morphogenesis occurred only in those regions of the limb (humerus, radius and ulna) that were forming during the excess gravity exposure. The rest of the limb developed similarly to controls, showing that suppression of limb morphogenesis requires continuous exposure to excess G. Biochemical determinations were carried out on lyophilized limbs exposed to 2.6, 3 and 3.6G. Exposure to these gravitational forces lowered the amount/μg dry weight of GAGs, but did not alter noncollagenous protein. Collagen was elevated in 2.6 and 3G limbs and decreased in 3.6G limbs.

To allow for culture of limbs as contralateral isolates, a new culture centrifuge was constructed, which carries eight multiwell tissue culture plates at three different radii, allowing experiments at three different G levels to be carried out simultaneously. As previously, the centrifuge is operated within a tissue culture incubator, allowing the use of standard culture conditions (37°C, 5% CO_2 and a humidified atmosphere). The centrifuge motor is outside the incubator to avoid any effects from ozone or electromagnetism.

Mouse palates have also been exposed to excess G in vitro. In initial studies 13- and 14-day palates were exposed to 2.6G for 24 hours. Sections for light microscopy were taken from the first, middle and third regions of the palates and scored as to the highest degree of fusion seen: 1-no fusion, 2- epithelial fusion, 3-epithelial breakdown, and 4-mesenchymal fusion. In later studies, serial transverse sections of palates were scored according to the stage (s) of fusion seen in each section. Chi-square analysis of the resulting frequencies in sections of 14-day palates showed a significant decrease in the frequency of no fusion in centrifuged palates and a significant increase in mesenchymal fusion in centrifuged palates.

For in vivo studies, a small animal centrifuge has been constructed. The centrifuge is based on the design of Walters, Wunder and Smith (1959) with two cage holders containing four cages each. When the centrifuge is operated at 40 and 45 rpm, the linear accelerations generated range from 1.8 to 3.5G (101-155 cm swingout radii). Two experiments have been conducted so far. In the first experiment, seventy female mice were placed in the centrifuge at four weeks of age, twenty at 1.8, 2.1 and 2.3G, and ten at 2.8G. Controls consisted of seventy female mice housed in the centrifuge room. The centrifuge was stopped 30 minutes every other day for ad libitum feeding of Purina laboratory chow, and maintenance, and 2.5 hours weekly for weighing. Water was available through a nuzzle valve system. After eight weeks of

centrifugation, mice were returned to 1G for two weeks. Since the mice were not housed individually, pairfeeding was not attempted. Mean weights of centrifuged mice were lower than controls at all time periods, significantly so for 2.1, 2.3 and 2.8 for weeks one to three, and for 1.8G mice throughout the experiment. During the experiment, mice in the top cage (1.8G) were found to weigh less than mice at higher G's. Since this could have been due to translucency of the top of the acrylic cageholders allowing the mice to orient on the overhead lights, the tops were covered 46 days into the experiment. When the cage tops were covered, the mice began to gain weight more rapidly; in two weeks their weights had surpassed those of mice at higher G's. Resposes of animals to return to 1G were varied. 1.8G animals were not affected; their rate of weight gain remained the same. Animals at 2.1 and 2.3G lost weight, and animals at 2.8G continued to gain. The second experiment was conducted similarly, using different G levels. The seventy four-week-old female mice were divided as follows: ten at 2.3, and twenty at 2.6, 2.9 and 3.5G. In this experiment mean weights of centrifuged mice were again lower than controls at all time periods. This difference was significant throughout the entire experiment for 2.6, 2.9 and 3.5G. An accidental return to 1G in this experiment (8.5 hour breakdown) resulted in loss of weight in mice exposed to 2.3 and 2.6G, while weight of mice exposed to higher G's (2.9 and3.5G) remained the same. These results, coupled with those obtained in the first experiment upon deliberate return to 1G, suggest that 1.8G mice were not unduly stressed by exposure to excess G nor by return to 1G. Animals at intermediate G's were stressed by return to 1G, resulting in weight loss. Animals at higher G forces were stressed by return to 1G, but were also released from some stress of exposure to higher G's.

For reproductive studies, mice were allowed to adapt to the excess G force for five weeks prior to breeding to avoid effects of maternal stress on the embryos. After adaptation, estrus was induced by replacing the bedding material in the female cages with bedding material from male cages. Two days later, 4-5 females from each G level were each paired with a male at 1G for 4 hours (9 AM-1 PM). (Males were not exposed to excess G). They were then returned to the centrifuge and sacrificed by cervical dislocation on gestational day 12 (Experiment I) and 18 (Experiment II). Embryos from both experiments were fixed in 3% glutaraldehyde in cacodylate buffer and crown rump lengths (CRL) measured. The number of pregnancies in control females was higher (5/15-Exp I; 4/16-Exp II) than in females exposed to excess G (3/20-Exp I; 1/16-Exp II), but there was no effect on litter size or number of resorptions/litter. Crown rump lengths (CRL) of embryos exposed to excess G were significantly lower than those of 1G embryos in all cases except at 1.8G. The smaller CRLs of centrifuged embryos cannot be explained simply by relation to smaller maternal size. In experiment I, 1.8G mice weighed less than the rest when matings were performed, but CRLs of these embryos did not differ significantly from controls.

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N85-26096

CELL DEATH, NEURONAL PLASTICITY AND FUNCTIONAL LOADING IN THE DEVELOPMENT OF THE CENTRAL NERVOUS SYSTEM

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The establishment of the complex central nervous system of mammals is an exercise in precision and plasticity: (1) precise timing of cellular birthdates from specifically designated generative sites, (2) precision migratory movements of newly produced neuroblasts from their generative sources, frequently over extensive distances through complex developing systems, to properly defined terminal structures with aggregates of like kind, (3) precise cytodifferentiation with suitably plastic expression of membrane properties, transmitter characteristics and cellular morphology dependent to an as yet unknown degree upon functional loading and finally (4) establishing proper but regulable [again apparently dependent upon functional loading] connectivity with relating structures and tracts. Failure in precision during any of these processes is believed to provide the groundwork for aberrant neural relationships that may result in death of the organism or unusual behavioral responsiveness to otherwise normal stimuli. However it has only been in recent years that the degree of plasticity in these precise sequential processes that appear to be dependent upon the functional loading of the specific system has been appreciated (e.g., Hollyday and Hamburger, 1977b; Boydston and Sohal, 1979).

This combination of both precision and plasticity in the development of the central nervous system strongly suggests potential for both evolutionary modifiability and phenotypic regulation dependent upon systemic requirements of the perinatal developmental environment. This is of paramount significance to gravitational biologists since, with the advent of space flight, exposure to gravitational vectors less then earth normal provide opportunities for modulation of the sensory loading of both vestibular and proprioceptive systems during selected developmental intervals.

Such regulation would require the systemic capability of modifying the responsive neuronal population. This may take the form of an elevation in the apparent rate of production of relevant neuroblasts, modulation of the phenotypic expression of the individual neuronal population during perinatal cytodifferentiation and maturation or modification of the systemic integration through synaptic field compression or expansion.

Any modulation in the responsive production of neurons would require changes in either the rate, length of time or site of production of neuroblasts. Such evidence has not yet been observed in any mammalian species. The mammalian central nervous system appears to have evolved a different mechanism to modulate the production of neurons in response to variations in functional loading through a process of regular overproduction of neuroblasts with subsequent degeneration of superfluous neurons during the process of perinatal maturation. Such cell death has been observed and commented upon for many years (Glucksmann, 1951; Prestige, 1970; Hamburger, 1975) but has only recently been positively correlated with changes in the functional loading of selected systems (see recent summary by Cowan etal, 1984).

Our research on the precise timing and regulation of neuron production and maturation in the vestibular and visual systems of Wistar rats and several inbred strains of mice (C57Bl6 and Pallid mutant) has concentrated upon establishing a timing baseline for mitotic development of the neurons of the vestibular nuclei and the peripheral vestibular sensory structures (maculae, cristae). This has involved studies of the timing and site of neuronal cell birth and preliminary studies of neuronal cell death in both central and peripheral elements of the mammalian vestibular system. Studies on neuronal generation and maturation in the retina have recently been added to provide a mechanism for more properly defining the 'in utero' developmental age of the individual fetal subject and to closely monitor potential transplacental effects of environmentally stressed maternal systems.

We will report on current efforts concentrating upon the (1) perinatal period of development (E18 thru P14) and (2) the role of cell death in response to variation in the functional loading of the vestibular and proprioceptive systems in developing mammalian organisms. We will also report preliminary results from rat fetuses flown on COSMOS 1514.

Our research contributes to the identification and understanding of:

(a) gravity dependent reproductive or morphogenetic events.

(c) developmental abnormalities attributable to weightlessness.

(d) how weightlessness affects tissue differentiation, organogenesis, and tissue/organ/system competence.

(f) how gravity affects maturation and plasticity of adult systems.

(g) the role gravity has played in the evolution of animal systems.

(h) effects of weightlessness on postnatal behavior and development.

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GRAVITATIONAL EFFECTS ON REPRODUCTION, GROWTH, AND DEVELOPMENT OF MAMMALS

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The broad objective of this research program is to determine the role which gravity plays in the growth and development of mammalian animals. Our current studies are focused on the effects of graded hypergravitational field intensities on mice, rats and other small sized laboratory animals using the chronic centrifugation technique. They include studies on reproduction and prenatal and postnatal growth and development. Among the important questions addressed are: 1) what stage or stages in animal development are affected by hypergravity and what are the effects? 2) is there a minimum or critical body size for hypergravity to produce a significant effect on growth and development? 3)are there field intensity thresholds for the preceding questions?

During the past year, our hypergravitational (hyper-G) studies have been redirected from previously used high-G intensities to the range between 1.0G and 2.1G. These lower G-intensities are more likely to be useful in assessing future space flight animal experiments involving fractional-G and zero-G than higher G intensities where high mortality rates are experienced by the newborn.

From analysis of the body masses at birth of rats conceived and allowed to undergo gestation under 2.1G and under normal gravity (1G), it was found that there was no significant difference between the two groups. Furthermore, their growth rates postnatally were the same until they reached a body mass of approximately 50 grams when the 2.1G group showed a significantly slower rate. Results from these studies support the conclusion that prenatal as well as the early postnatal stages of growth and development of the rat are refractory to hyper-G and suggest the real possibility that similar findings will be obtained in fractional-G and zero-G. Experimental studies on weanling rats and older aged rats having body sizes greater than 50 grams have shown that when exposed to 2.1G or lower hyper-G intensities, are very responsive exhibiting a marked decrease in their growth compared to 1G controls.

Additional studies on growth of mice under hyper-G conditions vs normal gravity lend further support to the conclusions reached in the rat studies. Weanling male and female mice subjected to 1.27G or 2.03G for 5 weeks attained the same body mass as control mice reared under 1G. The body mass of the males (ca 31g) and females (ca 23g) are well below the 50 gram body mass tentatively suggested as the minimum for growth responsiveness to hyper-G.

Inorder to further explore the questions posed above, fetal growth and development under various graded G-intensities are being determined in rats. Body mass and organ /body mass ratios at day 22 of gestation are being compared for fetuses exposed to 2.03G and 1.0G. Growth rate studies are also being performed on mice conceived and born under graded G-intensities between 1.0G and 2.03G. Similar reproduction and growth studies are to be initiated during the next year on the guinea pig. Fetal body mass of the guinea pig exceeds 50 g and offers an additional test of the proposed minimum body mass hypothesis.

ABSTRACTS - RESEARCH ASSOCIATES

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EVIDENCE THAT RESORPTION OF BONE BY RAT PERITONEAL MACROPHAGES OCCURS IN AN ACIDIC ENVIRONMENT

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Sponsor: Steven L. Teitelbaum, M.D.

Skeletal loss in space, like any form of osteoporosis, reflects a relative imbalance of the activities of cells resorbing (degrading) or forming bone. Consequently, prevention of weightlessness-induced bone loss may theoretically be accomplished by 1) stimulating bone formation or 2) inhibiting bone resorption. This approach, however, requires fundamental understanding of the mechanisms by which cells form or degrade bone, information not yet at hand.

An issue central to bone resorption is the pH at which resorption takes place. In this study, the pH dependent spectral shift of a fluorescent dye (fluorescein isothiocyanate) conjugated to bone matrix was used to determine the pH at the resorptive cell-bone matrix interface. Devitalized rat bone was used as the substrate, and rat peritoneal macrophages were used as the bone resorbing cells.

The pH at the cell-bone matrix interface is less than 6.0 at six hours incubation. The presence of the calcitropic agents, parathyroid hormone or calcitonin, has no effect on cell-matrix interface pH at six hours. Fibroblasts bind to bone without resorbing it; these cells do not generate an acidic environment at the attachment site.

The results suggest that bone resorption is the result of generation of an acidic microenvironment at the cell-matrix junction. Since the calcium-phosphate salts that comprise the major inorganic component of bone are soluble in acid, the generation of H^+ ion at the site of resorption probably results in removal of the inorganic component of bone. It also suggests that study of the enzymatic mechanism of degradation of the organic (principally collagen) phase of bone should be directed toward those enzymes which, unlike metalocollagenases, function in acidic environments.

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Circadian Rhythm Control: Neurophysiological Investigations

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(Sponsor: Dr. H. Craig Heller)

The suprachiasmatic nucleus (SCN) has been implicated as a primary component in central nervous system mechanisms governing circadian rhythms. Disruption of the normal synchronization of temperature, activity, and other rhythms is detrimental to health. Sleep-wake disorders, decreases in vigilance and performance, and certain affective disorders may result from or be exacerbated by such desynchronization. To study the basic neurophysiological mechanisms involved in entrainment of circadian systems by the environment, Parylene-coated, etched microwire electrode bundles were used to record extracellular action potentials from the small somata of the SCN and neighboring hypothalamic nuclei in unanesthetized, behaving animals.

Male Wistar rats were anesthetized and chronically prepared with EEG and EMG electrodes in addition to a moveable microdrive assembly. Single unit activity was recorded during electrophysiologically defined wakefulness, non-REM sleep (NREM), and REM sleep. Most units had S/N ratios > 3:1 which remained stable during movement and over time. The majority of cells had firing rates < 10 Hz and distinct populations of cells which had either the highest firing rate or lowest firing rate during REM sleep were seen. Sleep-state/firing rate profiles were similar to those seen in other brainstem areas thought to be important in the neural control of sleep. Further characterization of frequency, temporal patterning, and state-selectivity of SCN and surrounding hypothalamic nuclei will allow quantitative comparisons with CNS areas already explored. Future studies to elucidate the timekeeping properties of the SCN include (1) assessment of changes in responsiveness of photic SCN units over time; (2) characterization of thermal coefficients of SCN units; and (3) effect of anesthesia on SCN neuronal activity.

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THE EFFECTS OF SIMULATED WEIGHTLESSNESS ON SUSCEPTIBILITY TO VIRAL AND BACTERIAL INFECTIONS USING A MURINE MODEL

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Certain immunological responses may be compromised as a result of changes in environmental conditions, such as the physiologic adaptation to and from the weightlessness which occurs during space flight and recovery. A murine antiorthostatic model has recently been developed to simulate weightlessness. Using this model, the proposed study will determine if differences in susceptibility to viral and bacterial infections exist among mice suspended in an antiorthostatic orientation to simulate weightlessness, mice suspended in an orthostatic orientation to provide a stressful situation without the condition of weightlessness simulation, and non-suspended control mice. Inbred mouse strains which are resistant to the diabetogenic effects of the D variant of encephalomyocarditis virus (EMC-D) and the lethal effects of Salmonella typhimurium will be evaluated. Glucose tolerance tests will be performed on all EMC-D-infected and non-infected control groups. The incidence of EMC-D-induced diabetes and the percentage survival of S. typhimurium-infected animals will be determined in each group, and differences among the suspended and control groups determined. An additional study will determine the effects of simulated weightlessness on murine responses to exogenous interferon. The MM strain of encephalomyocarditis virus (EMC-MM) as well as the bacterium S. typhimurium are highly virulent in certain mouse strains, and are sensitive to protective effects of interferon. The ability of interferon to protect antiorthostatically suspended mice as compared to controls from these agents will be determined. If increased susceptibility to infection is established in the antiorthostatically suspended mice, additional studies will focus on specific and non-specific effector functions of the immune system.

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Participation of Ethylene in Two Modes of Gravistimulation of Shoots

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In shoots of many plants of which tomato is an example, ethylene production is increased during gravistimulation (Abeles, Ethylene in Plant Biology, Academic Press, 1973). Most of the evidence suggest that auxin and associated effectors mediate upward curvature and that ethylene retards it or participates in a counterreaction, but some recent publications suggest that ethylene may be a primary mediator of upward curvature. In order to elucidate the role of ethylene in gravitropism, detailed time courses for ethylene production in horizontal and upright plants were measured. Tomato and pea were chosen as examples of plants which exhibit different patterns of gravitropic curvature. In one pattern, the net rate of curvature is essentially constant until the shoot has become approximately vertical as seen in tomato. In the second pattern which is seen in pea, upon horizontal placement the stem begins to curve up rapidly, but soon this initial rate reverses at the tip and the extent of apical curvature decreases.

Tomato seedlings were placed in gas-tight lucite boxes from which air was sampled and analyzed for ethylene by GC-FID every 2 min for short-term studies and every 15 min for long-term studies. During the first 2 min interval after one set of plants was turned horizontal ethylene production was double the baseline. Similarly, plants rotated 3 rpm about a vertical axis transiently doubled ethylene production when the axis was shifted 90°. This immediate gravistimulated burst occurs with briefer lag than reported stimulation of net 1-aminocyclopropane-1-carboxylic acid (ACC) synthase activity. Thus, the transient burst of ethylene may be a direct result of gravity reception.

In order to clarify the role of this 2-min burst, the effect of exogenous ethylene was studied. Exogenous ethylene from 0.01 to 20 µl per liter air (µl/l) and the ethylene precursor, ACC, stimulated gravitropic curvature in tomato seedlings. The inhibitor of ethylene action, $AgNO_3$, and the inhibitors of ethylene biosynthesis, $CoCl_2$ and aminoethoxyvinylglycine (AVG), all retarded curvature. Therefore, ethylene may contribute to stimulation of gravitropic curvature in tomato seedlings.

In peas, epicotyls were excised, equilibrated for 4 h until wound ethylene had subsided to a low stable level, and ethylene production was mearured in vertical and horizontal segments placed in sealed vials. As for tomatoes, excised pea epicotyls increased their rate of ethylene production during the first 2 rin of gravistimulation. Also, very low concentrations of exogenous ethylene (0.5 to 5 nl/l) slightly enhance curvature. Thus, in pea, ethylene may play a role in early gravitropic curvature. On the other hand, higher levels of ethylene (0.05 µl/l and above) and ACC inhibit overall curvature. AVG was found to promote curvature. These results suggest that ethylene may indeed contribute to retarding curvature during the later stages (counterreaction) of gravitropic curvature.

Our major purpose is to examine the causes and effects of ethylene production during gravitropism. Therefore, we will next study whether ethylene production influences IAA redistribution in tomato and pea. Also, determination of shoot growth in the presence of ethylene will be examined.

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QUANTITATION OF Na^+, K^+-ATPase ENZYMATIC ACTIVITY IN TISSUES OF THE MAMMALIAN VESTIBULAR SYSTEM

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Sponsor: Dr. Dennis G. Drescher

The enzyme Na^+, K^+-ATPase is widely believed to play an important role in maintaining the unique, high-potassium ionic composition of endolymph. This fluid faces the hair-bearing surfaces of the various vestibular sensory epithelia, and its potassium content is indispensable for transduction by the mammalian hair cell. Because the endolymphatic compartment approximates a closed system, and the epithelial boundaries of the endolymphatic spaces are freely permeable to water, it is probable that the endolymph normally exists in a state of osmotic equilibrium with surrounding extracellular fluids. Thus, the ionic content of the endolymph will also be critical in controlling endolymphatic volume, since the ionic constituents of this fluid are almost solely responsible for its osmotic pressure. Inadequate regulation of endolymphatic volume is known to result in abnormal vestibular function. Transient variations in extracellular fluid volume and ionic composition, induced by first exposure to the microgravity environment, may disturb both the osmotic equilibrium and volume regulation of the endolymph; alterations of vestibular Na^+, K^+-ATPase activity may be involved in this process, and in subsequent recovery of homeostasis. In addition, Na^+, K^+-ATPase is likely to control the excitability of vestibular neurons, as it does in all other neuronal systems.

Studies reported the previous year (sponsored by Dr. S. A. Ernst and Dr. M. D. Ross) provided, for the first time, a detailed description of the enzyme's distribution in mammalian vestibular tissues. These investigations utilized the specific Na^+, K^+-ATPase inhibitor ouabain (^3H-labelled), for labelled-inhibitor autoradiographic localization of enzyme sites at the light-microscopic level. To demonstrate enzymatic activity at the electron-microscopic level, we utilized a cytochemical procedure in which the synthetic substrate p-nitrophenyl phosphate (NPP) is hydrolyzed by the enzyme, in a ouabain-inhibitable reaction, to yield as products nitrophenol (NP) and inorganic phosphate (P_i). P_i, precipitated at the site of enzymatic reaction by strontium ion in the incubation medium, was then visualized in the electron microscope. In vestibular structures of the albino guinea pig, Na^+, K^+-ATPase activity is highest in so-called "dark cells" of the utricular and ampullar walls, and is preferentially localized to basolateral membrane infoldings. This distribution is similar to that seen in other tissues where the enzyme is known to be involved in transepithelial transport of sodium and/or potassium ion; our results are consequently compatible with the hypothesis that the vestibular enzyme does, in fact, participate in the transport of potassium ion into endolymph. Vestibular nerve fibers represent a second major source of enzymatic activity.

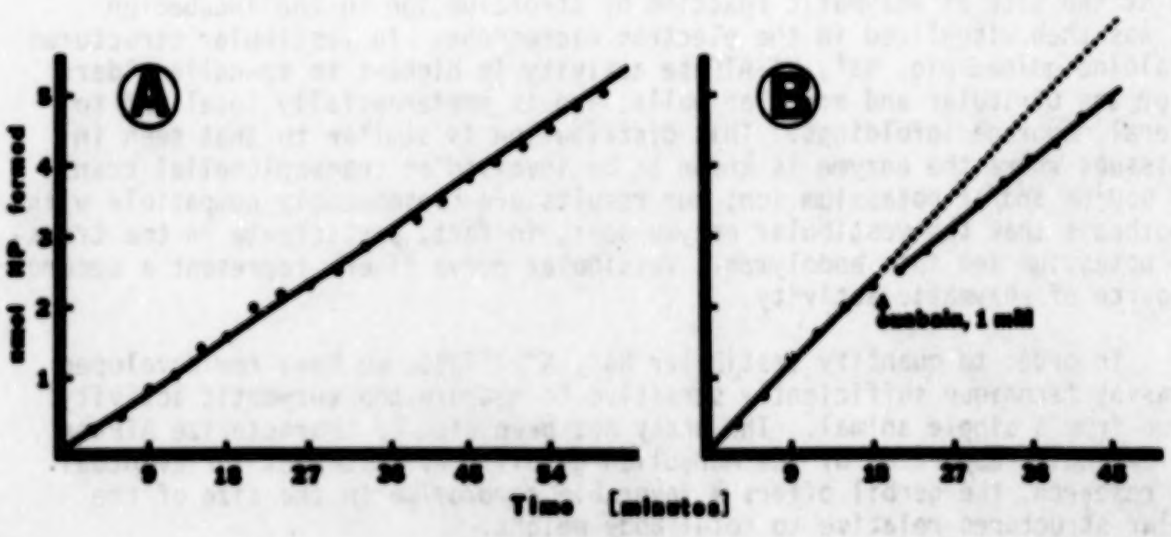
In order to quantify vestibular Na^+, K^+-ATPase, we have now developed a microassay technique sufficiently sensitive to measure the enzymatic activity in tissue from a single animal. The assay has been used to characterize ATPase in the vestibular apparatus of the Mongolian gerbil. As a subject for eventual orbital research, the gerbil offers a favorable compromise in the size of the vestibular structures relative to total body weight.

The standard tissue preparation utilized for these assays consists of the utricle (including the common crus), together with the ampullae of the lateral and superior semicircular canals. These structures can usually be dissected from the temporal bone in a single piece, and they account for all of the "dark cell" epithelial tissue in the entire vestibular apparatus, excepting that of the posterior ampulla. At the same time, this preparation excludes regions demonstrated by our earlier microscopical studies to be lower in enzymatic activity (e.g., the walls of the saccule and semicircular canals). Tissues from both temporal bones of a single animal are pooled. Histological examination of material thus prepared demonstrates that the integrity of the dark cell epithelium is maintained during the preparation procedure.

Like the cytochemical assay previously described, the quantitative procedure employs NPP (5 mM) as synthetic enzyme substrate. The quantitative assay, however, relies upon spectrophotometric measurement (410 nm) of nitrophenol (NP) released by enzymatic hydrolysis of the substrate. Product formation in the absence of ouabain reflects both specific (Na^+, K^+-ATPase) and non-specific (Mg^{++}-ATPase) enzymatic activity.

By measuring the accumulation of reaction product (NP) at three-minute intervals during the course of incubation, it is found that the overall enzymatic reaction proceeds linearly for at least 45 minutes (Figure 1, A). It is therefore possible to determine two separate reaction rates from a single set of tissues (Figure 1, B). Total ATPase activity is determined first; then ouabain (final concentration, 1 mM) is added to the incubation medium. The formation of reaction product after addition of ouabain represents non-specific (Mg^{++}-ATPase) activity. Reaction rates, normalized to dry tissue weight, are calculated from the slopes (nmol/hr) obtained during the first and second incubation periods. Na^+, K^+-ATPase (i.e., ouabain-sensitive) activity is equivalent to the difference in rates between first and second periods. Initial results indicate that total activity amounts to 53.3 ± 11.2 (S.E.M.) nmol/hr/mg dry tissue, of which approximately 20% is ouabain-sensitive. Effects of some agents with potential significance for vestibular function will be described.

FIGURE 1



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Isolation of hybridomas for Golgi-associated proteins and a plant calmodulin.

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Gravitropic stimulation results in differential growth in the upper and lower halves of horizontally oriented plant organs. Lee et al (1) have demonstrated that exogenous calcium can mimic gravitropic curvature while Roux and Slocum (2) have shown induction of calcium localization following gravitropic stimulation. The demonstration of a role for calcium in the mechanism of the gravitropic response indicates a role for calmodulin. Localization studies (3 - 5) indicate that plant cell walls have a high content of calmodulin which suggests a regulatory role for CaM in both gravitropic curvature and auxin-induced growth.

Auxin regulation of cell wall loosening and elongation is the basis for most models of this phenomenon. Auxin treatment of pea stem tissue rapidly increases the activity of Golgi-localized B-1,4-glucan synthase (GS), an enzyme involved in biosynthesis of wall xyloglucan which apparently constitutes the substrate for the wall loosening process.

In order to determine whether auxin stimulates GS activity either by modulation of existing enzyme or induces *de novo* formation of Golgi glucan synthase, we have undertaken to isolate and quantitate glucan synthase. This enzyme appears to be an integral protein of the Golgi membrane and has resisted isolation with retention of activity. The production of a monoclonal antibody for glucan synthase has been undertaken due to the inability to isolate GS by standard detergent/liposome techniques.

Currently, 32 hybridomas specific for Golgi-associated proteins have been identified by solid phase RIA and biotin/streptavidin sandwich ELISA. Although several antibodies exhibit some degree of inhibition of glucan synthase activity, none of the antibodies examined show complete inhibition. This suggests that the glucan synthase either represents a minor component of the Golgi proteins or that topologically, very little of the protein is available for induction of an immune response. To overcome this difficulty, hybridoma production by *in vitro* immunization, to circumvent auto-suppression, combined with immuno-suppresion of major antigens by cyclophosphimide is being used.

In order to specifically localize CaM and CaM-binding proteins, hybridomas directed against pea calmodulin have been produced. Currently, 68 MAbs are being characterized for epitope specificity prior to production of Fab' fragments.

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D50 N85-26104

SENSORY AND MOTOR PROPERTIES OF THE CEREBELLAR UVULA AND NODULUS

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Sponsor: Dr. D.L. Tomko

The uvula and nodulus (vermal lobules IX and X) of the vestibulocerebellum are implicated by behavioral evidence in the control of eye and head movements and in the production of motion sickness. The uvula and nodulus could play a role in these functions through known output pathways. Purkinje cells in both structures project via the fastigial and vestibular nuclei to the ventral horn of the cervical spinal cord (which controls neck muscles), to oculomotor neurons, and to the "emetic" region of the reticular formation (ablation of which abolishes susceptability to motion sickness). I plan to record from uvula and nodulus Purkinje cells in cats trained to make controlled head movements. I expect the activity of these neurons to modulate well during head and/or eye movements because the uvula and nodulus receive heavy projections from sources of visual (F.R. Robinson et al., J. Comp. Neurol. 223:471-482, 1984), vestibular and neck proprioceptive information. There is good evidence that single neurons in the uvula and nodulus receive input from several sensory modalities but nothing is known about what response properties result from this sensory convergence. By characterizing the sensory and movement related properties of these neurons I hope to determine how their activity contributes to movement and how different sensory inputs converge to influence this contribution. This work may also identify a population of neurons that modulates powerfully to the conflict between different head movement signals that can cause motion sickness.

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N85-26105

D51

TITLE: Differential Wall Growth in Gravistimuated Corn Roots
Its Timing and Regulation

Name: Bruce S. Serlin
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Sponsor: Dr. Stanley J. Roux

The experiments described in this proposal are designed to document cell-wall level changes which occur as a result of their gravistimulation. The goal of this research is to elucidate the mechanism and time frame of differential growth following a controlled gravistimulation. To achieve this, I will first ascertain rates of wall deposition by following the incorporation of radioactive monosaccharides into the wall. Complementing this experiment will be a freeze-etch study directed at revealing the spatial arrangement of both newly-deposited microfibrils and microfibrils that were present in the growing root prior to stimulation.

The second phase of the proposed research will examine the roles ethylene and Ca^{2+} have in the modulation of differential wall changes during gravitropism. Ethylene and Ca^{2+} have both been implicated as regulators of the gravitropic response in roots and they have also been reported to exert some control on the orientation of microfibrils. Both of these agents will be manipulated in such a way as to reveal whether they have a direct influence on cell wall deposition and microfibrillar alignment during the geotropic response.

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ANATOMY OF THE VESTIBULO-AUTONOMIC OUTFLOW TO THE GUT

N85-26106

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Sponsor: Dr. Robert H.I. Blanks

Motion sickness, a multisymptom disorder characterized by abnormal gastrointestinal motility and emesis, can be induced by vestibular effects on the sympathetic portion of the autonomic nervous system. However, the pathways linking the vestibular and autonomic pathways are unknown. As a first step in this analysis, the first series of experiments identified the location of preganglionic sympathetic neurons (PSN) and dorsal root afferent ganglionic neurons (DRG) which supply sympathetic innervation to major portions of the gastrointestinal tract in rabbit. Retrograde labeling of neurons was obtained by application of horseradish peroxidase (HRP) to the cut end of the greater splanchnic nerve. Labeled PSN are found, ipsilaterally, within T1-T11 spinal cord segments, with the highest density of neurons on T6. Most PSN are located within the intermediolateral column (IML), but a significant number also occurs within the lateral funiculus (LF), the intercalated area (IC) and the central autonomic area (CA). The proportion of labeling between the four areas depends on the spinal cord segment. Labeled cells in these four areas vary morphologically from large fusiform neurons in the IC to small fusiform neurons in the LF, small stellate neurons in the CA, and medium-sized stellate neurons in the IML. The DRG are labeled in thoracic segments T1-T12, with the majority located between T5-T11. These labeled DRG somata of the greater splanchnic nerve are smaller in comparison to unlabeled ones. In summary, the preganglionic sympathetic neurons projecting through the greater splanchnic nerve occupy a much larger functional domain than previously thought. The PSN are organized segmentally and posses a specific morphology in each region. The DRG innervating the viscera via the greater splanchnic nerve overlap the caudal spinal cord segments containing the PSN of the same nerve, have a wide distribution in size and are smaller than the total population. These findings should be considered in the future studies of the supraspinal and propriospinal control of visceral reflexes and of sympathetic outflow during vestibularly induced motion sickness.

Many brainstem nuclei have been implicated in the control of emesis. These include chemoreceptor area (area postrema), "vomiting center" within the medullary and pontine parvocellular reticular formation, motor nuclei which project to muscles involved in emesis (e.g., vagus, ambiguous and hypoglossal nuclei), sensory nuclei which receive information from the viscera and oral cavity (e.g., solitary tract

nucleus) and a diffuse set of nuclei which project down to the autonomic regions within the spinal cord (e.g., noradrenergic groups). The objective of the second series of experiments is to determine which of the brainstem nuclei project to the autonomic regions of the spinal cord that control gastrointestinal motility. To achieve this goal, a trans-synaptic retrograde tracer (3H-tetanus toxoid) is applied to the greater splanchnic nerve. This method allows the labeling of neurons within the brainstem that project only to the preganglionic sympathetic neurons.

One structure that has been strongly implicated in mediating vestibulo-autonomic control is the cerebellum (i.e., nodulus and uvula). The outflow of these lobules to the autonomic regions of the brainstem is mediated by the fastigial nucleus. To determine the precise projections of the fastigial nucleus to the brainstem nuclei involved in emesis, anterograde tracer (3H-leucine) was injected into the fastigial nucleus in a third series of experiments. Results show strong fastigal projections to the contralateral ponto-medullary parvocellular reticular formation (rostral "vomiting center"), lateral solitary tract nucleus and infratrigeminal nucleus.

The data of the second and third experiments give a complete description of one of the pathways involved in motion-induced gastrointestinal effects. These oligosympathetic pathways originating in the vestibular receptors, relayed by the vestibular nuclei, cerebellar cortex, fastigial nucleus and autonomic brainstem nuclei and terminating on preganglionic sympathetic neurons will be known. To further investigate the autonomic brainstem nuclei, a double-labeling experiment will be conducted. A fluorescent retrograde tracer will be injected into the preganglionic sympathetic nuclei and the brainstem tissue will be treated with glyoxylic acid to induce catecholamine fluorescence.

The results from these anatomical experiments have delineated the neurochemical and structrual connections between the cerebellum, autonomic brainstem nuclei and spinal cord sympathetic outflow to the gut. These studies will provide the basis for future physiological studies on the mechanisms underlying vestibular effects and the changes in these pathways during short- and long-term exposure to hypogravity.

OMIT TO END

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1. Report No.	2. Government Accession No.	3. Recipient's Catalog No.
NASA CP-2336		

4. Title and Subtitle	5. Report Date
NASA Space Biology Program: Annual Symposium	April 1985
	6. Performing Organization Code EB
7. Author(s)	8. Performing Organization Report No.
Thora W. Halstead, Chairman	
	10. Work Unit No.
9. Performing Organization Name and Address	11. Contract or Grant No.
Office of Space Science and Applications NASA Headquarters Washington, D.C. 20546	
	13. Type of Report and Period Covered
12. Sponsoring Agency Name and Address	Conference Publication
National Aeronautics and Space Administration Washington, D.C. 20546	14. Sponsoring Agency Code

15. Supplementary Notes

16. Abstract

Abstracts of research projects reported at the 9th Annual Symposium of the NASA Space Biology Program, held at Harper's Ferry, West Virginia, from November 6-9, 1984. The symposium program, abstracts of research, and a list of symposium participants are included. Topics covered included plant and animal gravity receptors and transduction, the role of gravity in growth and development of plants and animals, biological support structures and the role of calcium, mechanisms and responses of gravity sensitive systems, and mechanisms of plant responses to gravity.

17. Key Words (Suggested by Author(s))	18. Distribution Statement
Gravity, Plant, Animal Spaceflight, Weightlessness	Unclassified - Unlimited Subject Category 51

19. Security Classif. (of this report)	20. Security Classif. (of this page)	21. No. of Pages	22. Price*
Unclassified	Unclassified	138	A07

*For sale by the National Technical Information Service, Springfield, Virginia 22161

NASA-Langley, 1985

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DATE

FILMED

JUL 23 1985